Breakdown of plant tissues by pectic enzymes of fungal origin, with special reference to citrus fruits.

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BREAKDOWN OF PLANT TISSUES BY PECTIC ENZYMES OF FUNGAL ORIGIN, WITH SPECIAL REFERENCE TO CITRUS FRUITS.

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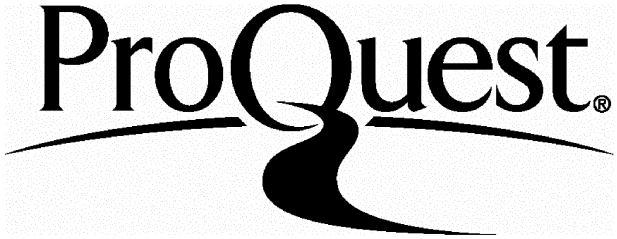

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SUMMARY.

The evidence for the participation of pectic enzymes in plant disease is reviewed, with particular reference to the part played by these enzymes in diseases of citrus fruits.

The pectic enzymes of Penicillium digitatum, a pathogen of citrus fruits were compared with those of Penicillium notatum a related species which is non pathogenic, giving evidence that pectin transeliminase and arabanase might be involved in pathogenicity. The pectic enzymes produced by P.digitatum and two other citrus pathogens, Penicillium italicum and Aspergillus fonsecaeus, under various growth conditions were investigated. An indication was thus given that , in the case of P.digitatum and P.italicum, pectin transeliminase was associated with maceration.

The pectin transeliminases of Penicillium digitatum and Penicillium italicum were purified till they were electrophoretically homogeneous. In such a state of purity, they were still found to be capable of macerating orange rind tissue. Evidence of another macerating system (possibly endo-polygalacturonase) was found in the case of P.italicum. Pectin transeliminase was not considered to play a significant part in maceration in the case of Aspergillus fonsecaeus, though the nature of the macerating factor produced by this organism is not known at present. Pectin transeliminase and macerating factor were shown to be present in orange tissue infected with P.digitatum or P.italicum.

The pectin transeliminases of Penicillium digitatum and Penicillium italicum were compared, and

were found to have the same electrophoretic mobilities. They were found to differ only in their susceptibility to temperature, and when injected into oranges they were found to cause areas of softening.

The significance of these results in the light of other work, is discussed; and suggestions are made for further research.

INTRODUCTION

The role of enzymes in host parasite relationships has long been established. Brown (1915) was one of the earliest workers to show this. He obtained an extract from germinated pathogen spores, and demonstrated that this extract was capable of destroying the coherence of any susceptible tissue placed in it. He noted that the action of the extract was firstly, to cause solution of the middle lamella so that the tissue lost coherence, and secondly, to cause the remaining layers of the cell wall to disintegrate, though complete solution of the cell was not observed. The extract was, he found, inactivated by heating thus suggesting its enzymic nature. In a later publication Brown (1936), suggested that although extracts from fungal pathogens were capable of causing disintegration of the host tissue, these extracts were not responsible for the primary invasion through the epidermis. Evidence for this view was obtained by injecting rose petals with an extract of Botrytis cinerea. After a quarter of an hour, the rose petals were destroyed, but the surface of the petals remained intact.

Plant Cellular Structure.

The nature of the enzymes produced by plant pathogens may be better understood by examining first the chemical structure of plant tissues. It will then be clear what substrates are available for such enzymes. Bailey (1938), has made a study of the physico-chemical composition of cell walls of higher plants. He distinguished three categories of cell structures as follows:-

1. Meristematic (embryonic) cells are capable of growth and have a wall consisting of cellulose and pectin.

2. Fibres and other differentiated cells, lose the potential for growth and retain their original primary wall in a modified form. They lay down a supplementary

wall with a mechanical function, consisting of cellulose with or without hemicellulose. This is known as the secondary wall and does not contain any appreciable fraction of pectic compounds.

3. Adjacent cells are held together by a truly isotropic substance of pectic nature. This intercellular layer is known as the middle lamella.

All these structures may become lignified with a corresponding reduction in pectin content. On the other hand soft tissues generally give high yields of pectic substances and low yields of hemicelluloses and ligin . Fruits and roots usually have a high pectin content, for example the peel of citrus fruits can contain from 20-40% pectin on a dry weight basis (Kefford 1959). The cell wall pectic substances are different from those in the intercellular layer, where they occur frequently as a salt with calcium. There are also a variety of other compounds associated with plant pectin which will be considered later.

It is evident that in order to cause disintegration of plant tissues, a pathogen must first cause dissolution of the middle lamella. These enzymes which break down pectic materials and associated compounds are likely to be involved in pathogenicity.

The Chemistry of Pectic Materials and Associated Compounds

The majority of early work on the chemistry of pectic substances was carried out by Hirst and Jones (1938, 1939, 1946). They isolated and analysed pectin and its associated compounds from apples, citrus peel, peanuts and lupins. Work on this subject has been reviewed extensively by Kertesz and McColloch (1950), Kertesz (1951), Whistler and Smart (1953), Deuel and Stutz (1958) and Joslyn (1962).

The pectin complex is not a definite structure, but all pectins so far isolated have yielded on hydrolysis

mainly the sugars L-arabinose and D-galactose, the uronide D-galacturonic acid and methyl alcochol. The central molecule in this complex is a polygalacturonide made up of D-galacturonic acid molecules, 1-4 α glycosidically linked in a linear fashion. The carboxyl groups of this polymer are either fully or partially esterified with methyl alcochol, and those which are not are sometimes involved in salt formation, in particular with calcium and magnesium. The araban, a polymer of L-arabinose and the galactan, a polymer of D-galactose, are associated with the polygalacturonide, and all three polymers are usually extracted together.

Whistler and Smart (1953) give a general classification of terms pertaining to pectic substances. This classification was laid down by a committee of the American Chemical Society as follows:-

1. Pectic Substances. This is a group name for those complex colloidal carbohydrate derivatives which occur in, or are prepared from plants, and contain a large proportion of anhydro-galacturonic acid units, which exist in a chain like combination. The carboxyl groups of the polygalacturonic acids may be partly esterified by methyl groups and partly or completely neutralized by one or more bases.

2. Pectic Acids. These are colloidal polygalacturonic acids, free from methyl alcohol, which form normal or acid pectinates as salts.

3. Pectinic Acids. These are colloidal polygalacturonic acids containing more than a negligible proportion of methyl ester groups. Under suitable conditions they are capable of forming gels with sugar and acids, or if low enough in methyl alcochol with certain metallic ions. Salts are either normal or acid pectinates.

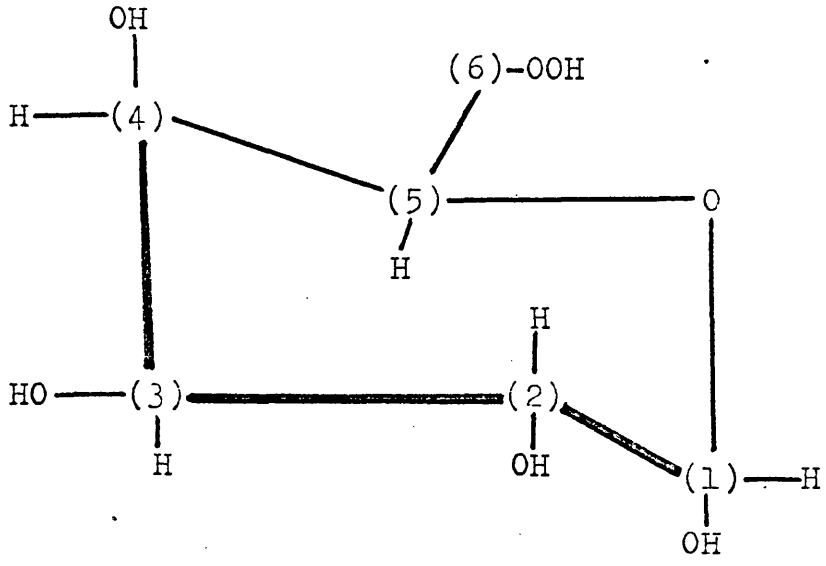
4. Pectins. These are water soluble pectinic acids of varying methyl alcohol content and degree of neutralization. They form gels with sugar and acids under certain conditions.

5. Protopectin. This is the water insoluble parent pectic substance, which occurs in plants, and which on restricted hydrolysis yields pectin or pectinic acids.

The term protopectin is now probably obsolete, as the insolubility of native pectin is most likely to be due to its association with other compounds. Before considering this however, the more precise chemical structure of the various parts of the pectin complex will be discussed.

The polygalacturonic acid is a linear polymer of D-galacturonic acid monomers, which due to the resistance of the polymer to hydrolysis, are likely to be in the pyranose form (Whistler and Smart 1953). This, together with the periodate oxidation technique has indicated that the monomers are joined by 1-4 glycosidic links, and enzyme hydrolysis experiments and the high optical rotation of pectic acid have suggested the α type of linkage. The polymer is a trans 1-4 polysaccharide, as the hydroxyl groups at carbon atoms 1 and 4 are in the axial position. Free rotation at the glycosidic linkages is therefore hindered and the pectin macromolecule may be considered a chain with restricted flexibility. Secondary hydroxyl groups at carbon atoms 2 and 3, and the carboxyl group at carbon atom 5 are in an equatorial position and are therefore easily accessible (Whistler and Smart 1953). X-ray diffraction of sodium pectate chains showed a threefold screw symmetry and chains are arranged in closest packing around the axis (Deuel and Stutz 1958). Molecular weights for pectic acid have been reported from 10,000 to 200,000.

The araban and galactan polymers are linked to the galacturonan partly by covalent links and partly by secondary binding forces. Thus pectin is part mixture and part compound. Araban is a pure polymer of L-arabinose, having a relatively low molecular weight. It is a chain of α1-5 linked arabofuranose units, half of which bear an



Form of Galacturonic Acid in Pectic Acid Chain showing Threefold Screw Symmetry (Deuel and Stutz 1958)

additional ∝ 1-3 linked L-arabofuranose residue as a one unit side chain. Araban is not found free from pectin in nature, though L-arabinose is commonly met as an integral portion of other polysaccharide molecules. The high negative rotation and the rapid rate of acid hydrolysis suggests that all arabinose units are in the furanose form and are connected by ∝ L-glycosidic linkages (Whistler and Smart 1953).

Galactan is a linear molecule made up of β 1-4 linked D-galactopyranose units, shown in part by its resistance to acid hydrolysis and its low optical rotation. The usual chain length for the galactan associated with pectin is about 100 D-galactose units (Whistler and Smart 1953)

Biosynthesis of Pectic Substances

The original theory that galactan was converted to pectic acid, which was then converted to araban is now discounted, due to the entirely different structures of the three polymers. Introduction of glucose-1-C 14, -2-C 14, -6-C 14 and galactose-1-C 14 into green strawberries through their stems, was carried out by Seegmillar, Jang and Mann (1956). After several days incubation the "pectin" fraction was isolated and hydrolysed to galacturonic acid and arabinose. The label in individual carbon atoms of the galacturonic acid and arabinose was determined by means of chemical degredation procedures. The results suggested that the intact carbon skeleton was preserved in the transformation of hexose to galacturonic acid. It also appeared that the intact chain of the first five carbon atoms of the hexose was preserved in the transformation of hexose to pentose (arabinose). U.D.P. appeared to be involved in this process (Deuel and Stutz 1958), though in fact UDP galacturonic acid has not been isolated from higher plants, only from Type I Pneumococcus which contains a polysaccharide rich in galacturonic acid. The methyl ester group is incorporated by trans-methylation using methionine, formate, or the α carbon of glycine or serine.

The Insolubility of "Native" Pectin

Isolated pectin is soluble to a greater or lesser extent depending on the degree of esterification, but native pectin is not. In considering the breakdown of plant material by enzymes from plant pathogens, it is of course native pectin that is of greatest interest. The term protopectin has been applied to insoluble plant pectin. This would now seem

to be a misnomer, as it suggests a substance different in basic structure from the pectins that have been isolated and analysed. It now appears more likely that it is the manner in which pectin and its asscociated molecules are bound in plant material that accounts for their insolubility. Joslyn (1962) has reviewed the various theories put forward to account for the insolubility of plant pectin, and it would be of value to summarize this review here.

The size of pectin molecules is known to have an effect on their solubility, in that large molecules are less soluble than smaller ones; and there has been evidence to suggest that this is the most likely factor involved in the anchorage of pectins in plant tissues. If this was a significant factor, then the native pectin would become noticably soluble on partial degradation, offering an explanation for the maceration of plant tissue by pectin depolymerases, which will be considered later. An extension of this argument is that plant pectin may be rendered insoluble due to the secondary aggregation of filamentous molecules of pectin with one another, or with large polymers in the cell wall. Aggregation has been explained as simply intertwining of the molecules, or possibly linking by bonds such as the anhydride, lactone or ester linkage. There is, however, much dispute over this last hypothesis, due to the different interpretations of the results of viscosity experiments.

The existence of a pectin cellulose compound has been suggested by several early workers in the pectin field, to account for the insolubility of native pectin. Ester formation between the free carboxyl groups of pectin and the hydroxyl groups of cellulose, or combination between the free carboxyl groups of pectin and the carboxyl groups of cellulose was put forward to explain this. The formation of ester or ether linkages

between pectin and hemicelluloses has also been suggested as an explanation for the insolubility of native pectins, on the evidence that araban and galactan are always found associated with pectin. Galactose and arabinose have also been found in the main uronide chain. Yet another theory is that salt linkages are formed between the carboxyl groups of pectin and the basic groups of proteins. Proteins have been identified in the middle lamella of some plants, and apple tissue proteins which are difficult to extract, are removed best under alkaline conditions which also remove most pectin. However, specific protein extracting agents such as thioglycollic acid and some proteases, appear to have no effect on the solubility of pectin in tissue preparations from certain varieties of apples.

The fact that the relatively small amount of pectin in lignified tissue is more difficult to extract than the pectin in non lignified tissue, led to the suggestion that an insoluble lignin pectin complex may exist. Although chemical combination between lignin and carbohydrates has been reported, the type of linkage of pectin and lignin that would decrease solubility is unknown and therefore such a complex is thought unlikely. Calcium, magnesium and iron have been demonstrated in the intercellular layer and in fact, highly insoluble double salts of galacturonic acid have been isolated. Also calcium pectinate and other polyvalent cation salts are well known for their insolubility. Thus the insolubility of "protopectin" could be explained entirely by pectin cation complexes. However the cation complexing reagents used to test this view, have been used under conditions that would in any case, lead to extensive denaturation and depolymerization of pectins. A great deal of further evidence is presented by Joclyn both for and against this view, but he was

not able to draw any definite conclusions as to which, of the several possible explanations for native pectin insolubility, was the correct one. The most likely explanation seemed to him to be that " Native pectin exists as a polygalacturonide, in which the hydroxyl groups on carbon atoms 4 and 5 are masked by glycoside and ring formation and the carboxyl on carbon atom 1 (5.e 5) is either free, esterified with methyl alcohol or esterified with araban, galactan or other polysaccharides. The hydroxyl groups on carbon atoms 2 and 3 may be free, esterified with acetyl groups, or linked by ether like linkages to polysaccharides or lignins. It is likely that non uronide sugars occur in the main chain as well as in branched chains. Ester, hemiacetal and ether linkage is suggested between chains of the polyuronide and polysaccharide. The degree of acetylation may also be a factor in determining the extractability of pectin."

Ginzburg, (1961) put forward evidence for a protein gel structure, cross linked by metal cations in the intercellular cement of pea root tips. Chelating agents aided the separation of the cells, which could then be recemented by treatment with divalent cations. Proteolytic enzymes, or denaturing agents stop the capacity to recement. Less importance was placed on the role of cations by McClendon and Somers (1960). They found that although chelating agents aided enzymic maceration of potato tuber tissue, they had no action alone, and calcium ions did not prevent maceration. Thus they concluded that, although calcium ions were present in this tissue, they did not contribute to its basic structure.

Further evidence for the importance of the non-uronide sugars in native pectin has been presented by Barrett and Northcote (1965), who achieved the separation of pectic substances of the apple into a pure,

weakly acidic pectic compound and a neutral arabinan (araban) galactan complex. The pectinic acid contained galacturonic acid, arabinose, galactose, rhamnose, xylose and several trace sugars linked covalently to the galacturonosyl residues. The suggestion was put forward that the galacturonosyl-(1-2)-rhamnose link is a general feature of pectinic acid structure. A similar situation was found in sycamore, but in addition, there was a strongly acidic polygalacturonic acid component. The galacturonsyl-(1-2)-rhamnose link has also been found in sycamore whole cambium.

An important point to be drawn out of the preceeding work, is that there is nothing which contradicts the view that, pectic enzymes may be an important factor in the breakdown of host tissue by plant pathogens. What this work does suggest however, is that there may be other enzymes working with, or as possible alternatives to, the pectic enzymes in tissue decomposition.

Enzymes Attacking Pectic Materials

The literature dealing with pectic enzymes is both prolific and confused. There have, however, been several excellent reviews on the subject, namely, Phaff and Joclyn (1947); Kertesz and McColloch (1950); Wood (1955); Demain and Phaff (1957); Denel and Stutz (1958); Wood (1960); and Bateman and Millar (1966). Pectic enzymes can be broadly classified into three groups. These are, firstly, enzymes removing the methyl groups from pectin, secondly, enzymes attacking the main uronide chain in one of several ways, and lastly, enzymes attacking the non uronide polymers associated with pectin.

The first group are known as the pectin methyl esterases (P.M.E.'s), and are found in a wide range of micro-organisms and plants. They have no effect on the main uronide chain. In plants they tend to be bound

strongly to the cell wall and have to be extracted by salt solutions (Jansen, Jang and Bonner, 1960). P.M.E.s of microbial origin, particularly those from fungi, generally show optimal activity at a lower pH than those of higher plant origin, and are less affected by the presence of salts in the reaction mixture (Bateman and Millar 1966). Jansen, McDonnell and Jang (1945) reported that polygalacturonase (P.G.), acting simultaneously with P.M.E. on pectin at pH 4.0 had a favourable effect on de-esterification and on the maintenance of the reaction rate, as compared with the action of P.M.E. alone. This suggests that P.M.E. is inhibited by its products. In sufficiently high concentrations, P.M.E. enhanced the action of P.G. on pectin to an extent that approached the rate observed on pectic acid.

The most important group of pectic enzymes consists of those enzymes attacking the main uronide chain of pectin or pectic acid. Attempts to classify these enzymes have been made by Demain and Phaff (1957) and Deuel and Stutz (1958), but due to the lack of knowledge about the recently discovered transeliminases, these classifications are now incomplete. Bateman and Millar (1966), have put forward the most comprehensive classification to date, and it is this that will be used here.

All the chain splitting enzymes have the ability to break the α1-4 glycosidic bonds between galacturonic acid monomers of the pectin or pectic acid chain. The main subdivisions in their classification depend on whether they attack such bonds by a hydrolytic mechanism, or by a transeliminative mechanism. Pectic enzymes of the hydrolytic type have been known for a considerable time, and split the glycosidic bond with the addition of water across it. The transeliminases are of more

recent discovery. The earliest report of such activity was by Albersheim, Neukom and Deuel (1960a). Using a commercial pectinase preparation, they found an enzyme which attacked only the methyl ester of pectic acid, giving breakdown products which absorbed strongly at 235nm., due to the presence of -4-5 unsaturated galacturonic acid groups. The mechanism of such action will be dealt with later. Hydrolases and transeliminases can then be further classified on whether they degrade methylated or demethylated pectin, and whether they attack the substrate in a random or terminal manner. Enzymes attacking the polymer chain in a terminal manner, ie. by removing monomers from the end, are known as exo-enzymes. Those attacking the polymer randomly are known as endo-enzymes. Using such criteria the classification of Bateman and Millar is as follows :-

A. Enzymes causing hydrolytic cleavage of the α 1-4 glycosidic links of pectic substances.

1. Random mechanism of hydrolysis.
    a. Pectin attacked in preference to pectic acid endo-polymethylgalacturonase (endo-P.M.G.).
    b. Pectic acid attacked in preference to pectin endo-polygalacturonase (endo-P.G.).
2. Terminal mechanism of hydrolysis.
    a. Pectin attacked in preference to pectic acid exo-polymethylgalacturonase (exo-P.M.G.).
    b. Pectic acid attacked in preference to pectin exo- polygalacturonase (exo-P.G.).

B. Enzymes causing transeliminative cleavage of the α 1-4 glycosidic links of pectic substances.

1. Random mechanism of transeliminative cleavage
    a. Pectin attacked in preference to pectic acid endo-pectin methyl transeliminase (endo-P.M.T.E. or P.T.E.)
    b. Pectic acid attacked in preference to pectin endo-polygalacturonate transeliminase (endo-P.G.T.E.)

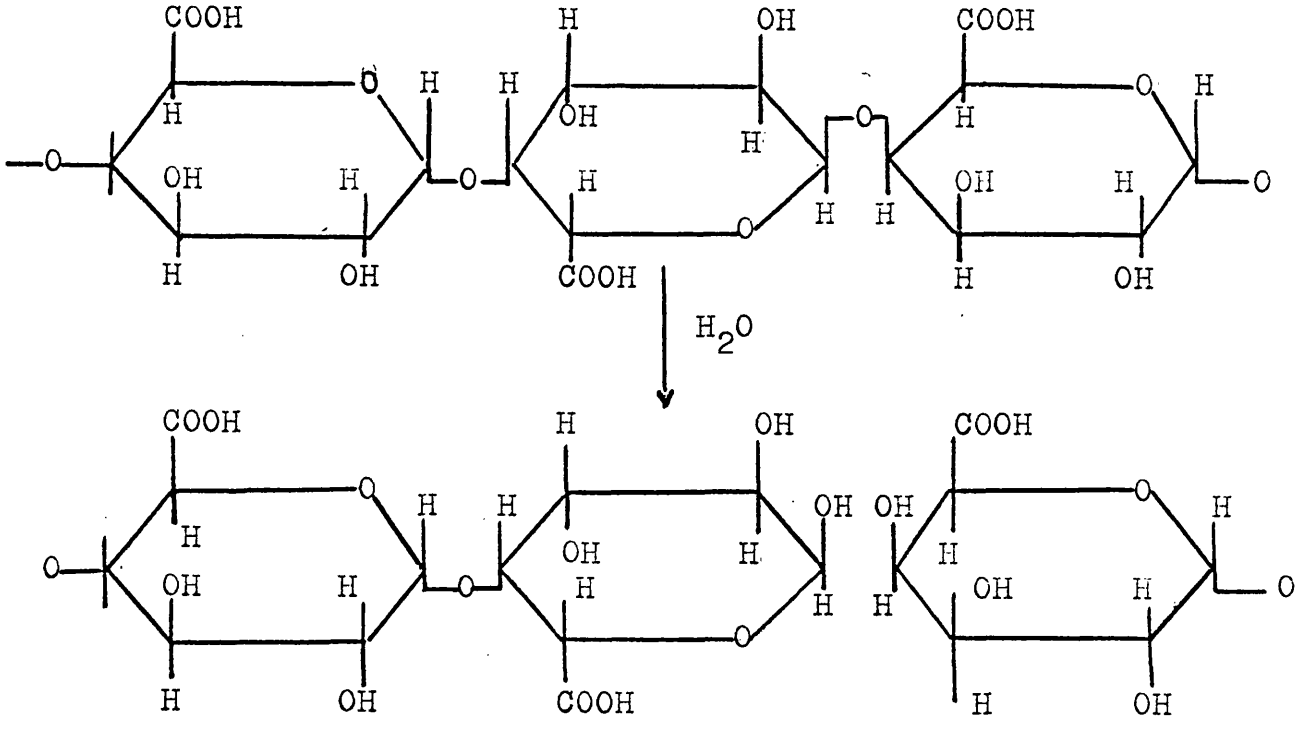
2. Terminal mechanism of transeliminative attack

a. Pectin attacked in preference to pectic acid exo-pectin methyl transeliminase (exo-P.M.T.E. or P.T.E.)

b. Pectic acid attacked in preference to pectin exo-polygalacturonate transeliminase (exo-P.G.T.E.)

Demain and Phaff (1957) used pH optima and extent of bond breakage to classify the enzymes further, but such criteria were not used by Bateman and Millar (1966) because they considered that there was too little information on the degradation of substrates by purified enzyme systems.

Hydrolytic pectic enzymes were familiar to some of the earliest workers in the field, but it is highly probable that many of the enzymes considered as hydrolases in the past were in fact transeliminases. The hydrolase attacks the 1-4 ∝ glycosidic bonds of the uronic acid polymers in the following manner.



Hydrolysis of part of a Polygalacturonide Chain

Pectic hydrolases are found in fungi, bacteria and higher plants. Luh and Phaff (1951a), demonstrated a P.G. free of P.M.E. in yeast cultures. They later showed that this enzyme caused incomplete breakdown of pectic acid to a mixture of digalacturonic acid and galacturonic acid, (Luh and Phaff 1951b, Demain and Phaff 1956). Although this was a three stage process, they showed that only one enzyme, endo-P.G., was responsible for it. Mill and Tuttobello (1961) identified and purified an endo-P.G. from Aspergillus niger. They showed that it acted on pectic acid in the same way as yeast P.G.. Patel and Phaff (1960) isolated a P.G. from tomatoes. They considered that the purified system consisted of two components, as digalacturonic acid was attacked, in contrast to the yeast and aspergillus enzymes.

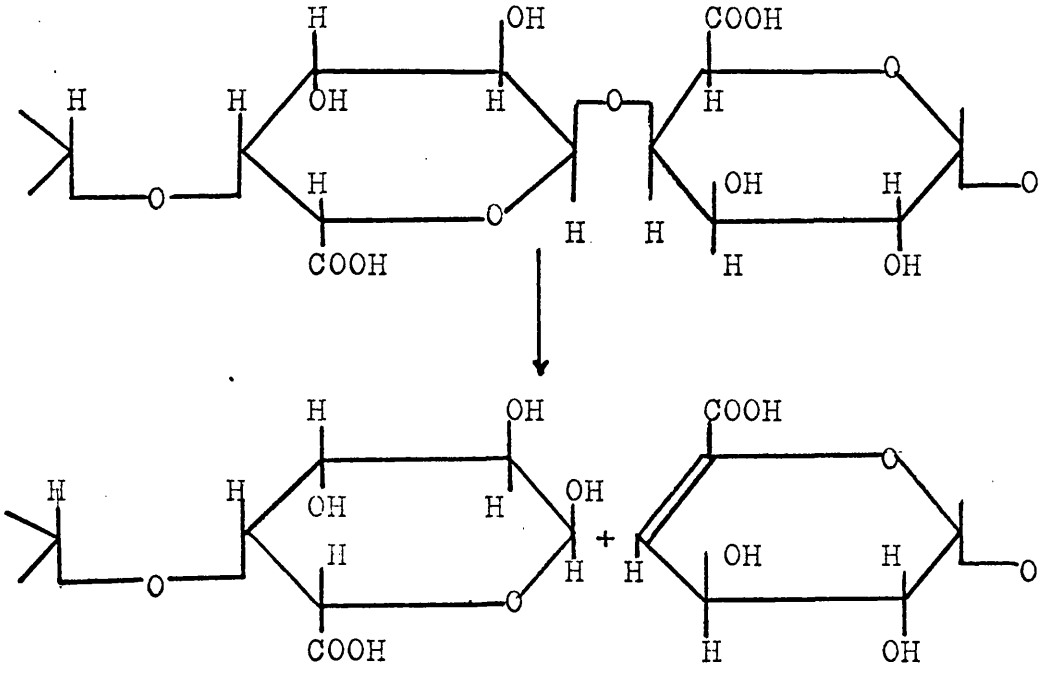
Perombelon and Hadley (1964) identified an endo-P.G. and an endo-P.M.G. in certain strains of Rhizoctonia and a similar situation was found in Erwinia carotovora by Masao Goto and Norio Okabe (1963). Akira Endo (1964) isolated and purified three endo-P.G.s from Coniothryum diplodiella. These had different optimum pHs. Apart from tomatoes, exo-P.G.s have been identified in several Botrytis species by Hancock, Millar and Lorbeer (1964), and in Botrytis cinerea by Barash et al (1964). Garber Beraha and Shaeffer (1965) also identified an exo-P.G. and an endo-P.G. in culture filtrates of Penicillium digitatum and Penicillium italicum.

A study of the alkaline breakdown of pectin (Neukom and Deuel, 1958) and the breakdown of pectin in neutral solutions (Albersheim Neukom and Deuel 1960b), showed that under these conditions the glycosidic links were cleaved by a β-dealkoxylation mechanism. This meant that the glycosidic linkage in the β position to

the ester carbonyl group of pectin was cleaved , after the removal of the activated hydrogen at carbon atom 5, and the formation of a double bond between carbon atoms 4 and 5. The products of such a breakdown showed an absorption maximum at 235 nm, and reacted with thiobarbituric acid to give a product which absorbed at 547 nm. There then followed the discovery that pectin and pectic acid could be broken down enzymically by this mechanism (Albersheim, Neukom and Deuel, 1960a). Investigations by Nagel and Vaughn (1961), on the degradation of oligogalacturonides by the P.G. of Bacillus polymyxa showed that this enzyme broke down trigalacturonic acid to give galacturonic acid and an altered dimer. This dimer absorbed strongly at 235 nm, and it was concluded that the enzyme was a transeliminase, rather than a hydrolase. It appeared to be an endo-P.G.T.E., as digalacturonic acid was not attacked. Starr and Moran (1961) identified a P.G.T.E. in culture filtrates of Erwinia carotovora and Bacillus polymyxa, while Macmillan and Vaughn (1964) and Macmillan, Phaff and Vaughn (1964), purified and studied the action of an exo-P.G.T.E. from Clostridium multifermentans. This enzyme was dependent on critical levels of certain divalent cations; calcium, strontium and manganese showing the greatest stimulation. It showed no activity on pectin, but removed units of O-(4-deoxy-β-L-threo-hexopyranos-4-enyluronic acid)-(1 → 4)-D-galacturonic acid (or α,β-unsaturated digalacturonic acid) from the reducing ends of polygalacturonic acid chains. Strangely enough, although it was an exo- enzyme, it did not attack digalacturonic acid.

Fuchs (1965) identified an inducible P.G.T.E. from three strains of Pseudomonas fluorescens. This enzyme was capable of converting sodium polygalacturonate into several reaction products, which strongly

absorbed light at 235 nm. Paper chromatography revealed that these products constituted a homologous series of oligouronides. The fastest moving compound appeared to be an unsaturated monouronic acid, 4-deoxy-L-threo-5-hexo-seulose uronic acid. This together with other indirect evidence, indicated that the enzyme was an endo-P.G.T.E. cleaving the glycosidic linkages as shown.



Transeliminative Cleavage of Part of a Polygalacturonide Chain.

A P.T.E. from a fungal source was purified by Albersheim and Killias (1962), taking advantage of the low isoelectric point of the enzyme (pH 3.5). After surveying several fungi, a study was made of the production and purification of a P.T.E. from Aspergillus fonsecaeus by Edstrom and Phaff (1964a and 1964b). They found that the enzyme caused a random cleavage of

pectin, giving α, β unsaturated reaction products, and that its action was enhanced by calcium ions. Another P.T.E. was isolated, together with a hydrolase, from cell extracts of an aerobic Bacillus (Hasegawa and Nagel, 1967). They separated the two enzymes, and found that the transeliminase attacked higher uronides in a random manner, but was unable to attack trigalacturonic acid. Again the enzyme was stimulated by calcium ions but unlike the transeliminase of Aspergillus fonsecaeus it was able to attack pectic acid as well as pectin.

The final group of enzymes involved in the breakdown of pectic substances, are those breaking down the non-uronide polymers associated with pectic acid and pectin. The most important of these are the arabanases and the galactanases. Kaji et al have identified an arabanase in a commercial enzyme preparation (1961) and also in the culture filtrates of several Aspergilli (1963). On purification, these enzymes appeared to attack the araban polymer in a terminal manner, by removing arabinose units from the end of the chain. In a later publication, Kaji et al (1964), found that the enzyme preparations could hydrolyse not only the 1:5 links of the main araban chain but also the 1:3 links of the branch chains.

The Role of Pectic Enzymes in Pathogenesis and Maceration

The enzyme responsible for breakdown of host tissue by pathogens was, for a long time designated as 'protopectinase'. The work of Joclyn (1962), quoted earlier, has indicated that protopectin is not a specific substance thus the existance of a protopectinase is unlikely.

Beavan and Brown (1949), attributed the macerating power of Byssochlamys fulva to a 'pectinase', which appeared to reduce the molecular size of pectin,

without the production of galacturonic acid. This enzyme was not found free of P.M.E., and it seems probable in the light of modern knowledge, that it was an endo P.G.. Similar P.G.s were isolated from Botrytis cinerea, Penicillium expansum and Aspergillus aureas, by Jermyn and Tom kins (1950). When a study of their action was made, they were found to hydrolyse pectic acid by a random scission, and to cause a rapid lowering of the viscosity of pectin solutions. It would seem likely that these preparations contained a mixture of P.M.E., P.G. and possibly P.M.G.. Such a situation was in fact verified by Jarvis (1953), who found two pectic enzymes in Botrytis cinerea, one acting on pectin and the other acting on pectin and pectic acid. Both enzymes caused viscosity reductions and the author claimed that they resembled protopectinase (macerating factor) more nearly than P.G.. It would seem that there is a confusion of terminology here. The P.G. refered to by Jarvis, is probably an exo-P.G. and the two viscosity reducing enzymes, endo-P.M.G. and endo-P.G.

Botrytis cinerea causes rotting of apples as do Sclerotinia fructigena and Sclerotinia laxa, but when Cole J.S. (1956) examined rotted tissue for pectic enzyme activity, he was unable to detect this. He was however, able to show that pectin had been broken down in rotted apple tissue, and he also found that oxidised apple juice had a pronounced effect in deactivating certain enzymes, particularly those produced by B. cinerea. This, he felt, was an adequate explanation of the absence of pectolytic activity in rotted apple tissue; though such enzymes were clearly involved in pathogenisis. Apple pathogens, causing a firm rot of apples, caused a reduction in the water soluble pectic substances of about 10 - 20%, whereas

soft rot fungi caused larger changes, of about 70% (Cole M. and Wood 1961a). Galacturonic acid, absent from sound tissue, was universally present in each type of rotted tissue. In a subsequent publication Cole M. and Wood (1961b), found that filtrates from S. fructigena and Penicillium expansum liberated galacturonic acid from apple fibre, which had been thoroughly extracted with cold water, leaving only the insoluble pectic materials. Substances claimed by these authors to be responsible for the inhibition of P.G. in healthy apple, were leuco-anthocyanins, changed to other compounds by the action of polyphenol oxidases.

Botrytis cinerea is pathogenic on a number of hosts and investigations have not only centered around its attack on apples. Leachates of Safflower blossoms contained nutrients that stimulated the germination of conidia of B. cinerea. Such leachates also contained pectic substances that induced the production of P.G. in germinating Botrytis conidia, according to Barash, Klisiewicz and Kosuge (1964). The P.G. activity was detected soon after the germ tubes appeared and increased as they elongated. Extracts of flower heads, infected with this organism, showed traces of galacturonic acid, not traceable in sound blooms.

In yet another host parasite relationship involving Botrytis cinerea, the production of pectic enzymes by this organism, together with Botrytis allii and Botrytis squamosa, was investigated by Hancock, Millar and Lorbeer, (1964a), using potato dextrose broth, detached onion leaves and intact leaves. P.M.E. and cellulase were found to be produced by all organisms under all conditions. B. allii produced trace amounts of P.G. in potato dextrose broth and both endo and exo-P.G. in detached and intact leaves; whereas B. cinerea and B. squamosa produced endo-P.G. under all

conditions, and exo-P.G. in detached leaves. When culture filtrates were sprayed onto onion leaves, the leaf spots induced were similar to those caused by the Botrytis species (Hancock, Millar and Lorbeer 1964b). Culture filtrates with little P.G. failed to cause leaf spotting even though they contained a moderate amount of cellulase. Assays of extracts from diseased onion plants indicated that endo-P.G. and P.M.E. were the principal pectic enzymes associated with Botrytis leaf spot. The absence of exo-P.G. from plants infected with B. cinerea and B. squamosa suggested to these authors that this enzyme did not play an important role in Botrytis leaf spot.

Sclerotinia sclerotiorum a pathogen of apples and other fruits, was shown to produce P.M.E., P.G. and macerating factor when grown on wheat bran (Echandi and Walker 1957). These activities were inhibited by various vegetable storage organ extracts (c.f. Cole M. and Wood 1961 a and b). Echandi and Walker claimed that Erwinia aroideae differed from S. sclerotiorum in that it produced no P.G. or P.M.E., but only a pectin depolymerase. Here again is an apparent confusion of terms, as the so called depolymerase was almost certainly an endo-P.G. or endo-P.M.G. or endo-transeliminase. E. aroideae was also investigated by Murant and Wood (1957), with reference to the rotting of potato tubers. A filtrate of this organism was also found to cause maceration of potato tuber tissue tissue, but this activity could be reduced by potato sap. The mechanism of resistance to infection by Erwinia, found in potato tuber tissue, has been investigated in detail by Lovrekovich, Lovrekovich and Stahmann (1967). In contrast to the results of Echandi and Walker (1957), Wood and Gupta (1958), detected both P.M.E.and P.G. in E. aroideae but they were unable to detect these enzymes in Pythium debaryanum, although this organism macerated potato tissue. P. debaryanum produced an enzyme causing

a reduction in the viscosity of pectin at a high pH which, it is now considered, was probably an endo-P.M.G. or an endo-P.T.E..

P.G. and P.M.E. were found in culture filtrates of Fusarium oxysporium and Fusarium lycopersici by Waggoner and Dimmond (1955). They claimed that P.M.E. plus P.G. caused maceration, and detected these enzymes in the vascular stream of tomato plants infected with Fusarium; thus indicating that these enzymes had a role in pathogenicity. In Fusarium solani and Rhizoctonia solani, Elasori (1958) correllated 'protopectinase' activity with enzymes that reduced the viscosity of pectin. He did this by comparing their pH optima. He found that the enzymes from both fungi were more active together than singly, and that only the Rhizoctonia enzyme formed galacturonic acid as an end product. The author concluded from these results that 'protopectinase' activity was probably due to a cumulative effect of several enzymes.

Later work on Rhizoctonia solani (Bateman 1963a), revealed that water extracts of bean hypocotyls infected with this organism, contained P.G. and P.M.E.. The P.G. complex produced by R. solani in bean medium, appeared to contain an exo-P.G. not produced in vivo, where only an endo-P.G. was present. The optimum pH for maceration by culture filtrates of this organism were found to vary, depending on the relative proportions of P.G. and cellulase (Bateman 1963b). From these experiments, he concluded that P.G. was primarily responsible for maceration, whereas cellulase appeared to play a secondary role. To confirm this he extracted P.G. from Rhizoctonia infected bean tissue, purified it and found that it macerated in the absence of P.M.E. and cellulase.

When extracts were made of bean hypocotyls infected with Rhizoctonia solani, they appeared to contain a dialysable inhibitor to P.G. and maceration (Bateman 1964). Calcium ions seemed to be most effective in this respect, having been detected by radio tracers around lesions. The suggested mechanism for this inhibition was, that the calcium ions formed calcium pectate with demethylated pectin, which was then resistant to attack by P.G.. Though the preceeding evidence favours strongly the involvement of the pectic hydrolases in Rhizoctonia host parasite relationships, Perombelon and Hadley (1964) could detect no larger amounts of endo-P.G., endo-P.M.G. and macerating activity in pathogenic Rhizoctonia strains than they could in non pathogenic strains. This suggested that factors other than pectic enzyme production and the ability to macerate host tissue may be involved in pathogenicity. Further to this, no P.G. or macerating activity was detected in cultures of the pathogen Phytophthora infestans, though P.M.E. was constitutive (Clarke 1966). The absence of pectic enzyme activity in this organism may be accounted for by the fact that it does not cause a soft rot disease. Nevertheless, it indicates that pathogenicity may not necessarily be dependent on the presence of pectic enzymes.

Evidence for the participation of pectic enzymes in other host parasite relationships, was found by Leal and Villaneuva (1962), who examined forty strains of Verticillium species grown on a pectin medium. High pectic enzyme activities were found in pathogenic strains and no activity was found in the non pathogenic ones. Similarly Abo el-Dahab (1964), using Xanthomonas malvacearum, found that by adding pectin

to a medium, the pectic enzyme production was increased and the cells became more virulent. Cellulolytic activity was not detected. Further evidence along these lines was put forward by Singh and Akhitar (1964) who found that the more virulent strains of Colletotrichum falcatatum produced more P.G. than the less virulent ones. Cellulase and P.M.E. were produced equally by all organisms. Evidence of a rather different nature for the involvement of pectic enzymes in rotting, was put forward by Edney (1964). He found that if apples were stored in 5% CO_2 and 3% O_2, rotting by Gleosporium species was considerably reduced. If the pathogens were incubated under these conditions, then the pectic enzyme production was reduced.

Wood (1955), in a review on pectic enzymes, concluded that the secretion of pectic enzymes by plant pathogens had two purposes. These were, firstly the breakdown of plant tissue and secondly further degradation of primary breakdown products to give substances, which would pass into the cell and be used for growth. Although at this stage he was uncertain as to the exact nature of these enzymes, the truth of his statement still holds, in the light of modern knowledge. Deuel and Stutz (1958) concluded that pectic enzymes were involved in the maceration of plant material and the killing of plant cells, though they did not commit themselves very specifically on the exact identity of the enzymes,and the extent to which they were involved. In a later review Wood (1960), admitted that the macerating activity, shown by some plant pathogens, followed the activity of the chain chain splitting pectic enzymes, but pointed out that with other organisms, the maximum activity of P.G. and P.M.E. were obtained under different conditions from

the maximum activity of maceration. In the same review Wood made the point, that to prove that an enzyme plays a part in plant disease, it must be shown to be present in diseased plant tissue. In conclusion he suggested that other factors such as proteases and arabanases, might have a part to play in maceration.

The main evidence for the involvement of arabanases in maceration and pathogenicity was obtained by Byrde and Fielding (1962 and 1965), These workers separated an endo-P.G. and a macerating factor from Sclerotinia fructigena and found that the latter liberated arabinose from potato tissue and also from a synthetic substrate, phenyl-α-L-arabinofuranoside. Further evidence for this view came from the work of Fuchs et al (1965), who detected highest arabanase activity in fungi attacking legumes, which have a high araban content. Extensive work by McClendon (1964), also produced a certain amount of evidence for the involvement of arabanases in phytopathogenicity.

No mention has yet been made of the role of the transeliminases in maceration and pathogenicity. Their discovery has increased the evidence for the general involvement of pectic enzymes in host pathogen relationships. Starr and Moran (1961), have shown that culture filtrates of Erwinia carotovora and Bacillus polymyxa, grown in pectin, had transeliminase activity. They later characterized the enzyme (Starr and Moran 1962), showing that it acted preferentially on pectic acid rather than pectin. It was activated by calcium ions to such an extent that E.D.T.A. produced complete inhibition. Dean and Wood (1967), fractionated culture fluids of Erwinia aroidea and found a close correlation between P.G.T.E. activity and maceration. When they incubated a sample of the enzyme with potato cell wall

material there was an increase in absorbance at 235 nm. This explains the results of Echandi and Walker (1957), mentioned earlier, who could find no P.G. or P.M.E. but only a depolymerase in filtrates of E. aroidea.

The main conclusions to come out of the work of McClendon (1964), apart from the possible importance of arabanases in pathogenicity, were that maceration could most likely be correlated with the endo forms of both hydrolases and transeliminases; and that exo-enzymes were unlikely to be involved in this process.

Calcium ions were found to stimulate the activity of a P.G.T.E. produced by Fusarium species grown on autoclaved alfalfa roots (Millar 1965). Calcium was also found to stimulate maceration of potato by culture filtrates containing P.G.T.E. but no endo-P.G. This contrasts directly with the results of Bateman (1964), who found that calcium ions inhibited P.G. and maceration in Rhizoctonia solani. Such a contrast is interesting, as it gives strong evidence to the view that maceration can be caused by both hydrolytic and transeliminative activities. A P.G.T.E., similar to that isolated from Fusarium, was detected in Colletotrichum trifolliі, Ascochyta imperfecta and Stemphylium botryosum, in the absence of endo-P.G. and endo- P.M.G., when these organisms were grown on autoclaved alfalfa shoots (Hankock and Millar 1965a). However only C. trifolliі produced P.G.T.E. in vivo in any significant amounts. This was associated with a greater change in pectic materials in the disease of alfalfa caused by this organism, than in the diseases caused by the other two. Although all three organisms were found to be capable of utilizing cellulose, only C. trifolliі produced this in vivo (Hankock and Millar 1965b). These authors also found certain evidence for

the involvement of proteolytic enzymes and xylanases in C. trifolli infections. The P.G.T.E. production was favoured by an increase in pH (Hankock 1965) and it was therefore thought to be significant that such pH increases took place during the course of pathogenisis of alfalfa tissues infected with C. trifolli

Futher evidence of the participation of transeliminases in pathogenesis, was obtained by Bateman (1966), who showed that Fusarium solani produced three pectic enzymes in vitro. These were, a hydrolase attacking pectin at pH 5·0, a hydrolase attacking pectic acid at pH 6·0 and a calcium dependent transeliminase, degrading pectin or pectic acid at pH 8·6 or above. The transeliminase only was produced on autoclaved bean hypocotyls, and it was the predominant enzyme in vivo. This work clarified the situation found by Elasori (1958), quoted above, in F. solani, where he identified pectin depolymerases distinct from P.G.s..P.G.T.E. has also been isolated from phytopathogenic bacteria in the genus Xanthomonas (Nasuno and Starr 1967).

Bateman and Millar (1966), have concluded, in their review of the subject, that maceration is not the property of a single enzyme, but that it could most likely be correlated with the endo forms of the pectic glycosidases (hydrolases) and lyases (transeliminases), as suggested by McClendon (1964). Again these authors found no evidence of maceration by exoenzymes. Although they found abundant circumstantial evidence for the involvement of pectic enzymes in pathogenicity, they felt that proof that the ability of an organism to produce pectic enzymes accounted for its pathogenicity was lacking.

The Pathogenicity of Penicillium digitatum, Penicillium italicum and Aspergillus fonsecaeus on Citrus Fruits.

Penicillium digitatum and Penicillium italicum cause the green and blue rots of citrus fruit respecively. The diseases are most prevalent after harvest, in the packing houses and in transit. Both organisms cause softening of the fruit and profuse sporulating growth appears over the surface. The spores of P.digitatum are olive green, whereas those of P. italicum are rather more of a blue green colour.

Klotz (1930), found that both Penicillium digitatum and Penicillium italicum grew inter and intracellularly in the albedo parenchyma, flavedo and epidermis; finally invading the juice vesicles. He noticed that the organisms produced a limited softening ahead of the invading mycelium and suggested that these macerating substances were for the most part intracellular; being secreted only in a limited quantity near the tips of the living hyphae. They were, he thought, liberated en masse from old, dead, autolysing mycelium in the regions of advanced decay. To test this theory, Klotz made a mycelial extract of the fungiin water and inserted this in cylindrical holes made in the albedo of the orange. The result was a distinct softening around the holes. This softening was darker and more pronounced than that caused by oxalic acid.

Green (1932), working mainly on the epidemiology of Penicillium digitatum and Penicillium italicum concluded that there was a resistance to infection in the rind of oranges, which could be broken down by acids and ammonium oxalate. Penicillia grown in citrus juice or rind, but not in synthetic media also appeared to be able to break down the rind resistance. Once this

system was broken down, infection could occur through the stomata in uninjured rind. Green admitted that this so called resistance destroying system might be enzymic. Further work on the epidemiology of these diseases indicated that susceptibility to infection wasto some extent, related to the mode of inoculation (Nadel Schiffman et al 1953). For example, inoculations pricked into the oil glands gave consistantly higher rates of infection than those between the glands. Kavanagh (1965) found that filtrates of Penicillium digitatum grown in a pectin medium, did not promote infection, whereas filtrates when glucose was the carbon source did. This worker demonstrated the production of P.M.E. and P.G. by P. digitatum in vitro, although he could find little cellulase. P.M.E. was demonstrated in greater quantity in 0·2M NaCl extracts of infected rind than in similar extracts of healthy rind. P.G. and cellulase were found to a slight extent in infected rind but not in healthy rind, though macerating activity, while produced in vitro, was not found in vivo.

Miyakawa (1962) found that both Penicillium digitatum and Penicillium italicum excreted P.M.E. and P.G. constitutively and also demonstrated P.G. activity in infected orange peel tissue. The macerating activity of the two moulds appeared to be lower than that of the less pathogenic fungus Penicillium expansum, suggesting to the author that pectic enzymes were not the sole factor involved in pathogenicity. However he found that both fungi could breakdown pectin and furthermore, use the products of such a breakdown as carbon sources. In a later publication Miyakawa (1963) suggested that D-galacturonic acid, produced by both P. digitatum and P.italicum on pectin media, might have an effect on pathogenicity. He found that galacturonic acid caused

in vitro maceration of orange peel. This compound also showed an activity in the promotion of infection by the two Penicillia, suggesting that D-galacturonic acid could cause a destruction of rind resistance. This observation contrasts directly with an observation made by Kavanagh (1965), that filtrates of P.digitatum grown in a pectin medium, did not promote infection. It is assumed that such filtrates contained galacturonic acid.

Cole A.L.J. (1967), in a very extensive survey of enzyme production by Penicillium digitatum in relation to the attack of oranges by this organism, found that it produced constitutively P.M.E., enzymes that reduced the viscosity of both pectin and pectic acid, P.T.E., cellulase, α-L-arabinofuranosidase, β-D-galactopyranosidase and macerating factor. On passing culture filtrates through Sephadex G75 he found that the peak of macerating activity coincided with the peak of the viscosity reducing enzymes and P.T.E.. The peak of the α-L-arabinofuranosidase was slightly different. A similar situation was encountered with culture filtrates of the organism, grown on pectin and sodium polypectate media, although less growth was obtained than in the medium without pectin. On a synthetic medium containing pectin, P.digitatum produced less viscosity reducing activity than before, though macerating activity was high. P.T.E. was also present on this medium and appeared to have the same optimum pH as macerating activity. No difference was found between pectic hydrolase, pectic lyase and macerating activity, by thermal inactivation experiments. Growth on a slightly altered synthetic medium led to a situation where P.T.E. was absent, but viscosity reducing activity and macerating activity were high. The

enzyme reducing the viscosity of pectin was found to lose some of its activity on freeze drying, whereas neither the enzyme reducing the viscosity of pectate nor macerating activity were affected by such a treatment. Dialysis appeared to reduce the activity of the viscosity reducing enzymes and maceration, none of which recovered after dialysis against salts.

Cole found that Penicillium digitatum, when grown on an orange albedo medium, produced P.T.E., pectin viscosity reducing activity and macerating activity, but no P.M.E.. There was a correlation between the loss of weight of an orange tissue preparation during degradation and the release of reducing groups from it. Galacturonic acid, galactose and arabinose were identified as end products of this degradation which occured in the absence of P.T.E.. Macerating activity and P.M.E. were detected in infected rind but viscosity reducing enzymes, P.T.E., cellulase and proteolase were absent. Cole found that macerating activity and pectin viscosity reducing activity were present in the juice of infected fruits, but P.M.E. and P.T.E. were not. Orange juice appeared to have a thermolabile inhibitor to macerating activity and viscosity reducing activity.

A comparison of pectolytic and cellulolytic enzyme production by Penicillium digitatum, Penicillium italicum and Penicillium expansum with enzyme production by avirulent mutants of these organisms, was made by Garber, Beraha and Schaeffer, (1965). Pectolytic and cellulolytic activity appeared to depend on the carbon source in the growth medium, even with the mutants. The avirulent forms of P.digitatum and P.italicum had pectolytic activity when they were grown on a pectin containing medium. Orange tissue rotted by P.digitatum and P.italicum had both pectolytic and cellulolytic

activity, though necrotic tissue, produced by avirulent mutants had only cellulolytic activity. In a later publication Garber and Beraha (1966), showed that endo- and exo-P.G. were produced by P.digitatum and P.italicum grown on a variety of media, whereas P.T.E. was not. These authors also found exo- and endo-P.G. in the tissue of oranges infected with these organisms.

A wide range of pectic enzymes have been shown to be produced by Penicillium digitatum and Penicillium italicum both in vitro and in infected fruits, thus indicating their role in the pathogenicity of these organisms. The abundant evidence quoted earlier, for the involvement of pectic enzymes generally in host parasite relationships, supports such an indication. However what still needs to be shown, is the exact nature of the enzymes responsible for the pathogenicity and more particularly, the macerating activity of P.digitatum and P.italicum. Further studies on the enzymes produced both in vitro and in vivo by these organisms; and fractionation of culture filtrates of them,may help to discover the identity of the factor causing maceration. Such studies may also reveal to what extent pectic enzymes are involved in the rotting of citrus fruits by Penicillia.

A fungal disease of citrus fruits known as the 'black rot' has been reported by Fawcett, (1926). This is not as prevalent as the Penicillium rots and is normally only found in countries with a very hot climate. The organism causing such a disease is Aspergillus niger. A wide range of pectic enzymes have been shown to be produced by this mould. Tuttobello and Mill (1961), identified an endo-P.G., Kaji et al (1963) an arabanase and Edstrom and Phaff (1964), purified a P.T.E. from Aspergillus fonsecaeus, a species of the

A.niger group. It was decided therefore, to investigate the pectic enzymes produced by A.fonsecaeus and to compare them with the enzymes produced by the Penicillia mentioned above, in order to determine whether the same factors were responsible for pathogenicity and maceration in each organism.

EXPERIMENTAL PART 1. Materials and Methods.

All chemical used, were supplied by British Drug Houses Ltd., unless otherwise stated. ANALAR reagents were used wherever possible, especially in the analytical techniques.

a. Growth and Maintenance of Cultures

Penicillium digitatum Saccardo was obtained from a mouldy orange and isolated on malt agar. Penicillium notatum Wehmer, Penicillium italicum and Aspergillus fonsecaeus (Aspergillus niger 41875) were obtained from the Bath University of Technology fungal culture collection. P.digitatum and P.notatum were maintained on malt extract agar (3% Oxoid malt extract + 0·5% Oxoid mycological peptone + 1·5% Oxoid agar in water), whereas P.italicum and A.fonsecaeus were maintained both on this medium and on Czapek Dox agar in Universal bottles.

In order to obtain large spore crops for inoculation of liquid or bran cultures, 5ml. of a sterile 0·05% Tween 80 solution were added to a Universal bottle culture. After gentle shaking by hand, 1ml. of the spore suspension so formed, was pipetted aseptically into a 20oz. medical flat, containing a 5mm. layer of malt extract agar. The bottle was manipulated so that the spore suspension covered the entire surface of the agar; and was incubated at 25°C till profuse sporulating growth was apparent. In order to harvest the spores, about 20 sterile glass beads size 7 - 8mm., and 50ml of a 0·01% sterile Tween 80 solution were poured aseptically into the medical flat, which was then agitated vigorously to suspend the spores. The number of spores per ml in the suspension

was determined by means of a Thoma haemocytometer cell and an appropriate amount of suspension could then be used to seed either liquid or bran cultures. Liquid shaken cultures were grown in 250ml flasks containing 75ml of medium, on either a New Brunswick gyrotatory shaker or a Gallenkamp orbital incubator at 25°C, using a 1" throw at 330 rev/min. Liquid surface cultures were also seeded in 250ml flasks, containing 75ml of medium and were incubated at 25°C in a constant temperature room. Bran cultures were likewise grown at 25°C in a constant temperature room, in either 250ml or 1 litre conical flasks, depending on the amount of bran used (10g or 40g respectively, moistened with an equal amount of water).

The filtrates of liquid cultures were harvested by filtering through Green's no. 904 paper, in a Buchner funnel. The bran 'filtrates' were obtained by adding water to the bran (100ml of water to 10g of dry bran), and allowing this to stand for an hour. At the end of this time, the bran was removed from the liquid by filtering through muslin and the filtrate was centrifuged at 2500 rev/min (2075 g) for 1 hour in a M.S.E. Mistral 6 litre centrifuge. After harvesting, filtrates were either frozen immediately, or freeze dried in an Edwards Model 30 centrifugal freeze drier.

b. Ammonium Sulphate Fractionations

Partial separation of the enzymes in culture filtrates was effected by fractional precipitation with ammonium sulphate. The precipitate formed after each addition of ammonium sulphate was removed and redissolved. The amounts of ammonium sulphate to be added to a solution to give the desired degree of saturation, were obtained from a nomogram by Dixon (1953). Additions

were always made to solutions at refrigeration temperatures (4°C or less). After the requisite amount of ammonium sulphate had been added to a culture filtrate, this was left to stand at 4°C for 15 minutes to allow all the protein be precipitated. The solution was then centrifuged at 12,000 rev/min (23,000 g) for 20 minutes in an M.S.E. 18 high speed centrifuge, and the supernatant was removed. The precipitated protein was then dissolved in cold water and either stored in a deep freeze at - 25°C, or used immediately.

c. Column Chromatography

1. Sephadex. Columns of Sephadex G25 were used for desalting enzyme solutions, prior to their passage through ion exchange cellulose. The Sephadex was prepared by first immersing it in distilled water (wt./vol. ratio 1/10) for three days to allow the gel particles to swell. A column 30cms by 1·8cms was then poured from this slurry, containing 10g dry weight of Sephadex, in the following way. The column was filled to one third of its height with distilled water, the drain tap having been closed. The suspension of Sephadex was then poured into a separating funnel which fitted, via a ground glass joint, into the top of the column. The funnel tap was then opened, followed by the drain tap at the bottom of the column. In this way Sephadex flowed into the column as water flowed out and packing of the gel was completely even. The top of the gel was protected from disturbance by placing on top of it, a piece of filter paper, with the same diameter as the inside of the column. Samples were applied to the top of the column and were eluted with distilled water.

2. Ion Exchange Cellulose. The cellulose used in this work was Whatman's ET 11 ion exchange cellulose for column chromatography, often known as ECTEOLA cellulose. It was prepared for use according to the manufacturer's instructions and the method of Byrde and Fielding (1962). 25g of cellulose were stirred into 500ml of water and allowed to stand for 30 minutes. At the end of this time the supernatant was decanted off and the process was repeated, resulting in the removal of very fine particals which would hinder column flow. The cellulose was then suspended in 500ml of 0·5 N HCl for 1 hour, after which it was filtered under suction. Washing with water then followed until the pH of the washings collected in the filter flask was between 4 and 5. The cellulose was then suspended in 500ml of 0·5 N NaOH for 1 hour, followed by washing as before, until the pH of the washings was between 6 and 7. The purpose of the acid/alkali treatment was to impart sufficient hydrodynamic force to the ion exchanger, in order to break the hydrogen bonding in the vicinity of the ionic groups. Such hydrogen bonding is induced by drying. The cellulose was then suspended in 500ml of 0·5 M KH_2PO_4 to remove U.V. absorbing impurities, washed again in water and finally in 0·01 M phosphate buffer pH 7·5. It was then suspended in distilled water prior to column preparation. From 25g of cellulose, a column 45cms by 2·5cms was prepared. The cellulose suspension was poured into the column which was half filled with water, and at the same time the drain tap at the bottom of the column was opened to allow the water to flow through, thus ensuring even packing of the cellulose. The top of the column was protected from disturbance by a filter paper disc, in the same way as

the Sephadex. Desalted samples were applied to the top of the column and elution was carried out, using water and a stepwise salt gradient. Fractions from column chromatography were collected on a Central fraction collector and frozen or used immediately the run was completed.

d. Dialysis

Salts were sometimes removed from enzyme samples by dialysis, using a 1" Visking dialysis tubing supplied by the Scientific Instrument Centre Ltd.. 100ml of sample were dialysed against two changes of 5 litres of distilled water at $4^{\circ}C$.

e. Conductivity Measurements

Salt concentration was measured in enzyme samples by means of a Mullard conductivity bridge, made by the M.E.L. company. Samples with conductivities of less than 5×10^2 μ mhos were subjected to ion exchange chromatography.

f. Thin Layer Chromatography

The apparatus of Shandon and the media of Merck were used in all the thin layer chromatography carried out in this work.

1. Chromatography of Sugars. The thin layer method of Stahl and Kaltenbach (1961), was used to separate sugar monomers found as a result of the action of culture filtrates on orange tissue. Chromatoplates were prepared by spreading a mixture of 30g of Kieselguhr G. and 60ml of 0·02 M sodium acetate (shaken for 1 minute) in a layer 0·3mm thick. The plates were dried for 1 hour at $100^{\circ}C$ and stored in a container with silica gel to protect them from moisture. Chromatograms were run in an ethyl acetate:isopropanol:water (64:24:12)

solvent system and when dry, they were developed by spraying with aniline hydrogen phthalate and heating for 5 minutes at 105°C. The aniline hydrogen phthalate was made up by dissolving 0·96g of aniline and 1·62g of phthalic acid in 100ml of water saturated butanol. This spray distinguished pentoses by a pink colour and hexoses by a brown yellow colour.

2. Chromatography of the Breakdown Products of P.T.E. Acting on Pectin. The thin layer method of Ovodov et al (1967), was used to separate the products of the action of P.T.E. on pectin. Chromatoplates were prepared by spreading a mixture of 30g of Silica Gel G. and 60ml of 0·07 M NaH_2PO_4 in a layer 0·3mm thick. Plates were dried and stored as before. The chromatograms were run in an ethanol:butanol:0·1 M phosphoric acid (10:1:5) solvent system and when dry they were developed by spraying with conc. H_2SO_4 in ethanol (1:1 v/v), followed by heating for 5 minutes at 100°C. This reagent detected organic material by a charring effect, therefore galacturonic acid and all of its oligomers right up to pectin would be detected.

g. Arabanase Assay

Arabanase was assayed in culture filtrates by measuring the rate of release of arabinose from an araban substrate under defined conditions

1. Preparation of Araban. Preliminary experiments indicated that araban could be obtained in greatest yield from sugar beet. This was therefore used as a source and extracted by the method of Jones and Tanaka (1965). 3·3kg of molassed beet pulp nuts, kindly supplied by the British Sugar Beet Corporation, were soaked in running water for 24 hours to remove the molasses. The remaining beet pulp was then suspended

in 15 litres of distilled water, to which had been added 100g of calcium hydroxide and steam was blown through this mixture for 24 hours. At the end of this time the material was filtered through muslin and centrifuged at 2,000 rev/min (1320 g) for 30 minutes in a M.S.E. Mistral 6 litre centrifuge. The resultant supernatant was concentrated to 3 litres by means of a Quickfit climbing film evaporator and freed from calcium salts by precipitation with oxalic acid. This solution was then freeze dried in an Edwards Model 30 centrifugal freeze drier, giving a cream coloured powder which, on analysis was found to contain about 50% araban. This material proved to be a satisfactory substrate for the arabanase assay and was therefore used without further purification.

2. Measurement of Arabinose. 1ml of enzyme was added to 3ml of a 1% solution of the araban in citrate phosphate buffer pH 3·9 and this was incubated for one hour at 36°C in Universal bottles. These conditions were determined by experiments to be recorded later. The reaction was stopped by boiling for 15 minutes. The reaction mixture was diluted 10 times to measure its arabinose content, which was done by the method of Tracey (1950). In this method, 6ml of reagent made up by mixing 16ml of colourless aniline with 100 ml of glacial acetic acid followed by the addition of 24ml of distilled water and 3ml of 5% oxalic acid, was added to 2ml of diluted reaction mixture and left for 24 hours. The resultant colour was measured at 470 nm on a Unicam SP 600 spectrophotometer and was converted to µg/ml arabinose by reference to a standard line. A unit of arabanase activity was defined as that amount of enzyme causing the release of 1µg of arabinose per minute under the conditions stated.

h. Pectin transeliminase Assay.

Pectin transeliminase was measured by the method of Albersheim and Killias (1962), with certain modifications. 0·1ml of enzyme was allowed to act on 3ml of 0·5% Kodak citrus pectin solution in 0·1 M citrate phosphate buffer at pH 5·2. The reaction was carried out in a 1cm silica cuvette at room temperature, and the increase in U.V. absorption at 235 nm was recorded. In the published method, the substrate was clarified by means of an enzyme preparation, but in the present work it was found to be more convenient to clear the substrate by centrifuging at 18,000 rev/min (40,000 g) for one hour at 4°C. One unit of transeliminase activity was that amount of enzyme which caused an increase in optical density at 235 nm of 0·01 optical density units, in one minute, under the conditions described above. Samples were assayed for pectic acid transeliminase activity by this method, using pectic acid (0·5%) in buffer at pH 5·2, instead of pectin.

i. Assay of Reducing Group Liberating Enzymes.

P.G. activity was measured by determining the amount of reducing groups liberated from pectic acid, according to the method of Jansen and McDonnell(1945). Pectic acid was washed in 70% ethanol to remove any traces of reducing sugar impurities and was then dissolved in 0·1 M acetate buffer pH 5·0 to give a 0 5% solution. The pH of the solution was adjusted to 5·0 with 1 N NaOH. To 15ml of this substrate was added 0·5ml of enzyme, and after mixing, 5ml was immediately withdrawn and pipetted into 0·9ml of M Na_2CO_3 in a glass stoppered bottle. The enzyme substrate mixture was incubated for 8 minutes at 30°C and a further 5ml

was withdrawn and pipetted into 0·9ml of M Na_2CO_3 in another glass stoppered bottle. 5ml of 0·1 N iodine were then added to each bottle and left for 20 minutes at the end of which time 2·0ml of 2 N H_2SO_4 were added and the mixture was shaken. The contents of each bottle were then titrated against 0·05 N sodium thiosulphate. From the difference in titration values, the number of milli equivalents of iodine used, and consequently the number of milli moles of reducing group liberated could be calculated, having ascertained that one milli equivalent of iodine = 0·530 milli moles of reducing group. The P.G. unit of activity is then defined as the amount of enzyme which causes a liberation of 1 μ mole of reducing group per minute under the conditions described above.

j. Assay of Viscosity Reducing Enzymes

Enzymes reducing the viscosity of pectin were assayed by the method of Roboz, Barratt and Tatum (1952), using 0·5% pectin in citrate phosphate buffer pH 5·2 as a substrate. Those reducing the viscosity of pectic acid were assayed using 2·0% pectic acid in acetate buffer pH 5·0 to correspond with the reducing group assay. 0·25ml of enzyme was added to 7·5ml of substrate and the time noted. 4ml of this enzyme substrate mixture were then pipetted into a viscometer immersed in a water bath at 25°C, and at recorded time intervals the time for the liquid to fall between two points was determined, using a 0·1 second stop watch. The viscosity of the substrate + boiled enzyme and the buffer + boiled enzyme was also measured as controls. The percentage decrease in viscosity was calculated by the following formula:- $A = \frac{Vo - Vt}{Vo - Vs} \times 100$

where, A = percentage decrease in viscosity.

Vo = flow time in seconds of the substrate + the heat inactivated enzyme.

Vt = flow time in seconds of the substrate + the enzyme, at recorded time intervals.

Vs = flow time of the buffer + the heat inactivated enzyme.

A unit of viscosity reducing activity can then be defined as that amount of enzyme, which causes a decrease in viscosity of 1% under the conditions described.

k. Plate Assays for P.M.E. and P.G.

The methods used were those developed by Dingle, Reid and Solomons (1953).

1. P.M.E. The agar contained Kodak citrus pectin (1%), salicylanilide (0·01%) and agar (2%) dissolved in water to 100%. The pH was adjusted to 6·0 with NaOH. After autoclaving, sufficient methyl red in ethanol was added to the solution to give a final concentration of 0·01%. The pH was then readjusted with NaOH if necessary to discharge any trace of red colour of the indicator. The molten agar was poured into Petri dishes in layers 3mm thick and when it had set, holes were cut in it with an 8mm cork-borer. 0·01ml of enzyme solution was added to each well and after 20 hours incubation P.M.E. activity was detected by the presence of a red zone around the well.

2. P.G. the agar contained sodium polypectate (1%), prepared as described below, salicylanilide (0·01%), ammonium oxalate (0·7%) and agar (2%) dissolved in 0.2 M acetate buffer pH 5·0 to 100%. The medium was autoclaved at 15 lbs/in^2 pressure for 15 minutes and poured into Petri dishes in layers 3mm

deep. Holes were cut in the agar with an 8mm cork-borer and 0·1ml of enzyme solution was added to the well. After 20 hours incubation at 25°C, P.G. activity was detected by the presence of a clear area surrounded by a white halo around the well, after developement with 5 N HCl.

Though it is claimed that the zone diameter varies linearly with the log. of the enzyme concentration, these tests were only used qualitatively in the present work.

The sodium polypectate used in the P.G. assay was prepared from Kodak citrus pectin by the method of Jermyn and Tom kins (1950). 100g of pectin was stirred with a mixture of 375ml of 95% (v/v) aqueous ethanol and 125ml of N NaOH twice for 20 minutes. It was then washed with a mixture of 200ml of 95% ethanol 20ml of water and 10ml of N HCl. Finally it was dried in an oven at 37°C. The product was the sodium salt of pectic acid.

1. Assay of Macerating Activity

Macerating activity was assayed by placing discs of orange rind cut with a 9mm cork-borer into solutions of enzyme for 1 hour at room temperature. Maceration was scored at the highest possible dilution on the following arbitrary scale.

±	very slight softening compared with the control.
+	softening
++	extreme softening
+++	partial disintegration
++++	almost complete disintegration

m. Protein Estimations

Protein was estimated by the method of Lowry et al (1951). This involved the reaction of the phenol groups in tyrosine and tryptophan with the Lowry reagents to give a blue colour that could be measured spectrophotometrically. The reagents used were made up as follows:-

Reagent 1. 2% sodium carbonate in 0·1 N NaOH.

Reagent 2. 0·5% copper sulphate ($CuSO_4.5H_2O$) in 1% sodium tartrate $(CH(OH).COONa)_2 2H_2O$

Reagent a. was made up by adding 50ml of 1. to 1ml. of 2.

Reagent b. was standard B.D.H. Folin and Ciocalteau's reagent diluted in the proportion of 10ml of reagent to 18ml of water.

To 1ml of protein sample in a boiling tube was added 4ml of reagent a., and the mixture was immediately shaken. After 10 minutes 0·4ml of reagent b. was added to the mixture and the tube was shaken and left for at least 30 minutes. The blue colour which developed could then be read on a Unicam SP 600 spectrophotometer against a reagent blank at 750 nm. Absorption values were converted to µg of protein by reference to a standard line, using bovine serum albumin in concentrations of 10 to 280 µg/ml.

n. Electrophoresis

Fractions were examined for electrophoretic homogeneity by disc electrophoresis on polyacrylamide gels at pH 9·5, according to the technique of Davis and Ornstein (1964), using the apparatus of Shandon. Gels were loaded with about 250ug of protein and run at 5 m.a. per tube. They were stained with Naphthalene Black 12 B to locate the protein bands and the gel was destained in 7·5% acetic acid.

o. Estimation of the Methoxyl Content of Pectic Substances

Methoxyl estimations were done by the method of Nanji and Norman (1926), which involved first a calibration of the method with methanol and then the measurement of the methanol released from a sample of pectin or pectic acid. 100ml of potassium permanganate solution (9·75g/l) and 40ml of NaOH (150g/l) were introduced into a 500ml Quickfit conical flask. To this mixture was added the dilute methanol solution containing not more than 10mg. The mixture was well shaken and boiled under a reflux condenser for three minutes. 100ml of oxalic acid (20g/l) were then added to this without cooling, followed by 40ml of H_2SO_4 (conc. acid to water, 2:5). After shaking, the excess oxalic acid was titrated against 0·05 N K_2MnO_4 (1·58g/l). A control experiment was carried out without the addition of methanol. By using at least 5 methanol solutions of known concentration, it was possible to obtain a standard curve of methanol against the volume of permanganate used.

Methanol was released from pectin by treatment with sodium hydroxide. To a 0·5% solution of the pectin or pectic acid, containing not more than 0·05g of the substance, was added 100ml of 0·1 N NaOH in a glass stoppered flask. This was left overnight, after which it was neutralized to pH 4·5 with 0·5 N H_2SO_4, transferred to a distillation flask and distilled until approximately nine tenths had distilled over. The methanol estimation was then applied to the whole distillate as described before.

EXPERIMENTAL PART 2. Results.

a. Determination and Verification of Reaction Conditions for Enzyme Assays.

1. Arabanase. The method used for the assay of arabanase depended upon the measurement of arabinose released from an araban substrate. Since this method has not been published, it was necessary to determine the conditions for the assay, as described in 'materials and methods'. The pH and temperature optima were estimated approximately at first; then using these conditions an assay of enzyme activity over a period of time, at two substrate levels was carried out. The results are shown in Fig 1. and from them it was decided to use a substrate level of 1% araban and an incubation time of 1 hour for the assay.

Using these conditions and the approximate pH optimum, the reaction was studied over a range of temperatures. The results of this experiment can be seen in Fig 2.. The enzyme appeared to be almost inactive below about 5°C and above 50°C. The optimum temperature for enzyme activity was between 34 and 38°C. Results were not consistant enough to obtain a more accurate value than this, so it was decided to carry out the assay at 36°C. The effect of pH on enzyme activity was then examined at a reaction temperature of 36°C and the results are shown in Fig 3. The enzyme appeared to be active in the range pH 2·5 to 6·5 with a fairly sharp optimum at about pH 3·9.

In order to check whether, under these conditions, an increase in activity really represented a proportional increase in enzyme concentration, a sample of culture filtrate, containing arabanase was

Fig 1. Activity Time Curves for Arabanase at Substrate Levels of 8 and 10 mg/ml.

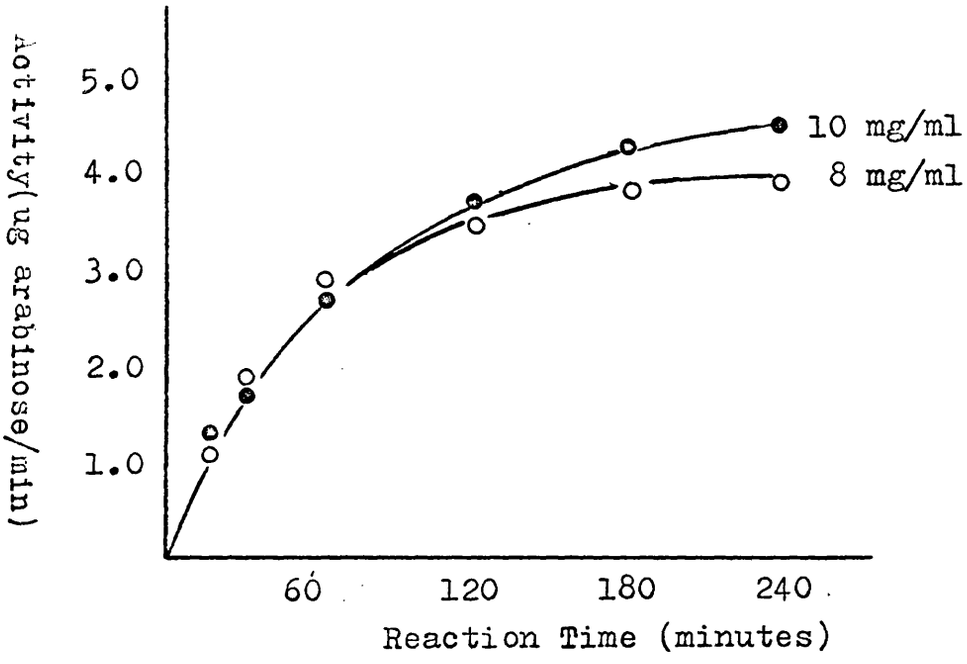


Fig 2. Activity Temperature Curve for Arabanase.

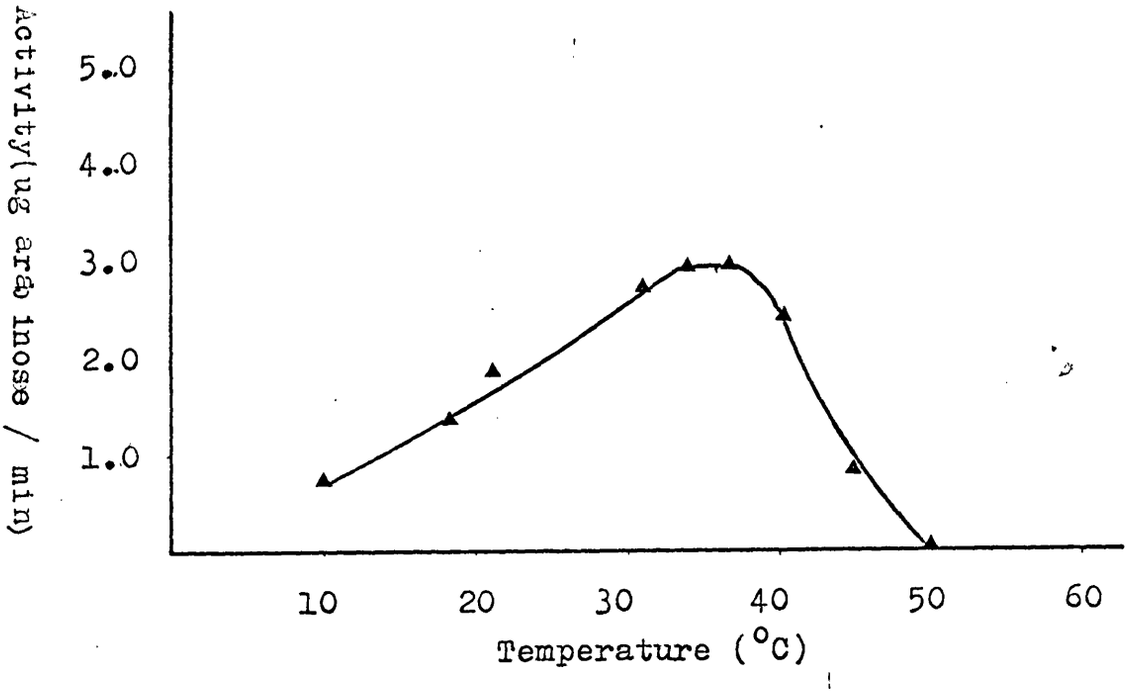


Fig 3. Activity pH Relationship of Arabanase.

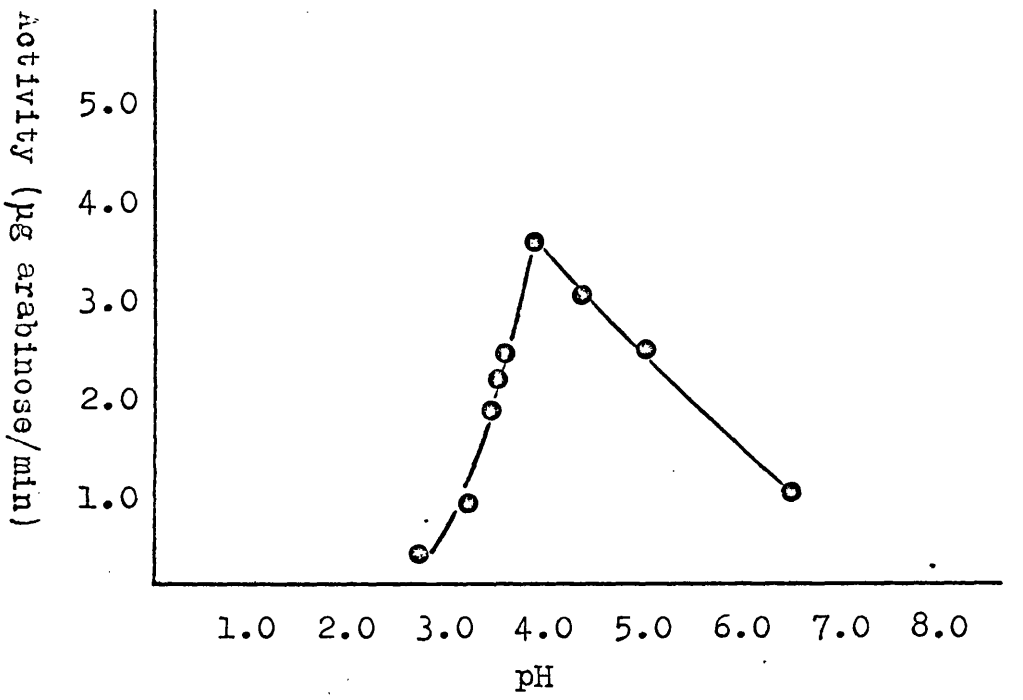
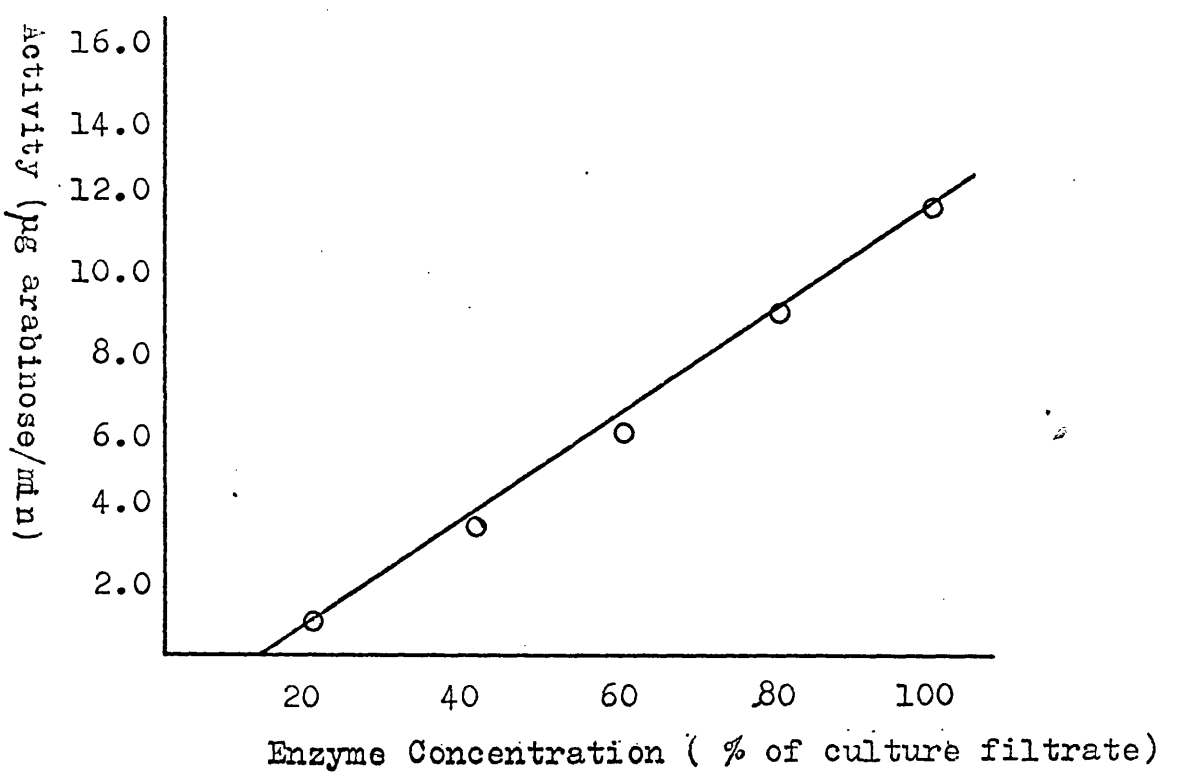


Fig 4. Enzyme Concentration : Activity Relationship of Arabanase.



diluted to give the following concentrations:- 80%, 60%, 40%, and 20% . A sample of each concentration was then assayed for arabanase activity. The results of this experiment are shown in Fig 4. and verify that over the range tested (0 - 12µg arabinose/min.), activity was proportional to enzyme concentration.

2. Pectin transeliminase. This enzyme was assayed by the method of Albersheim and Killias (1962), with only minor modifications. In order to determine a suitable reaction time for the assay, the reaction was followed spectrophotometrically as described in 'materials and methods', over a period of time. Dilutions of a sample of culture filtrate containing this enzyme were made, in order to demonstrate a straight line relationship between enzyme concentration and activity. The results of these experiments are shown in Figs 5 and 6 respectively. At 10 and 15 minutes reaction time, chosen from Fig 5. it was found that enzyme concentration was proportional to activity over the range tested.

3. Reducing Group Releasing Enzymes. Enzymes causing the liberation of reducing groups from pectic acid were measured by the method of Jansen and MacDonnell (1945). This was a modified Willstatter - Schudel hypoiodate method, depending on the reduction of iodine by the reducing groups liberated from pectic acid during the course of its breakdown. The method was first calibrated by obtaining a standard curve with galacturonic acid (Fig 7.), making use of the facts that 1 milli mole of galacturonic acid produces 1 milli mole of reducing group; and that 1 milli equivalent of iodine = 10ml of 0.1 N iodine. The values of milli moles of reducing group to milli equivalents of iodine , that were obtained using galacturonic acid concentrations from 0.5 to 10.0 mg/ml

are shown in table 1.

Table 1. Relationship between Milli Moles of Reducing Group from Galacturonic Acid and Milli Equivalents of Iodine.

Galacturonic Acid Concentration (mg/ml)	Milli Moles of Reducing Group	Milli Equivalents of Iodine
10	0.2577	0.410
8	0.2062	0.367
6	0.1546	0.287
5	0.1288	0.246
4	0.1030	0.195
2	0.0516	0.097
1	0.0258	0.049
0.5	0.0128	0.023

Taking values which fell on the linear portion of the curve (Fig 7.), it was found that 1 milli equivalent of iodine was reduced by 0.530 milli moles of reducing group.

A suitable reaction time for the assay was determined using a fungal enzyme preparation,known as Carterzyme. This preparation, kindly supplied by Beechams Food and Drink Division of Coleford, contained a P.G. which had previously been shown to release reducing groups from low methoxy pectin. This enzyme was incubated with buffered pectic acid under the conditions stated previously, in order to determine a suitable reaction time for the assay. The reducing group liberation was then plotted against reaction time as shown in Fig 8. From this experiment it was

decided to use a reaction time of 8 minutes for the reducing group assay. In order to check that activity was proportional to enzyme concentration, a series of dilutions was prepared from a sample of Carterzyme. The activity of each dilution was then assayed and activity was plotted against concentration. The concentrations used were, 100, 80, 60, 40, and 20% solutions of the Carterzyme. The results of this experiment are shown in Fig 9., indicating that concentration was proportional to activity over the activity range 0 - 2 μ moles reducing group/minute.

4. Viscosity Reducing Enzymes. The method for estimating viscosity reducing enzymes used in the present work, was that of Roboz, Barratt and Tatum (1952), with certain modifications as indicated in section 2. (materials and methods). The assay was carried out on 0.5% pectin at pH 5.2 and on pectic acid(2.0%) at pH 5.0, to correspond with the transeliminase assay and the reducing group assay respectively. The reaction with both substrates was followed over a period of time in order to determine a suitable reaction time. The results of one such experiment are shown in Figs 10 and 11. From these results it was clear that the viscosity reducing activity fell off rapidly after a period of only one or two minutes. It was therefore decided to run the assay routinely over 4 minutes, taking readings every minute. In order to check whether reduction in viscosity over this period could be used to measure the amount of enzyme present, samples of enzyme were diluted to 80%, 40%, 20% and 10% of their original concentrations and these diluted samples were assayed for enzyme activity, by measuring the reduction in viscosity produced in 1 minute. The percentage decrease in viscosity was then plotted

Fig 5. Increase in Absorption at 235 nm of a Pectin Solution due to P.T.E.

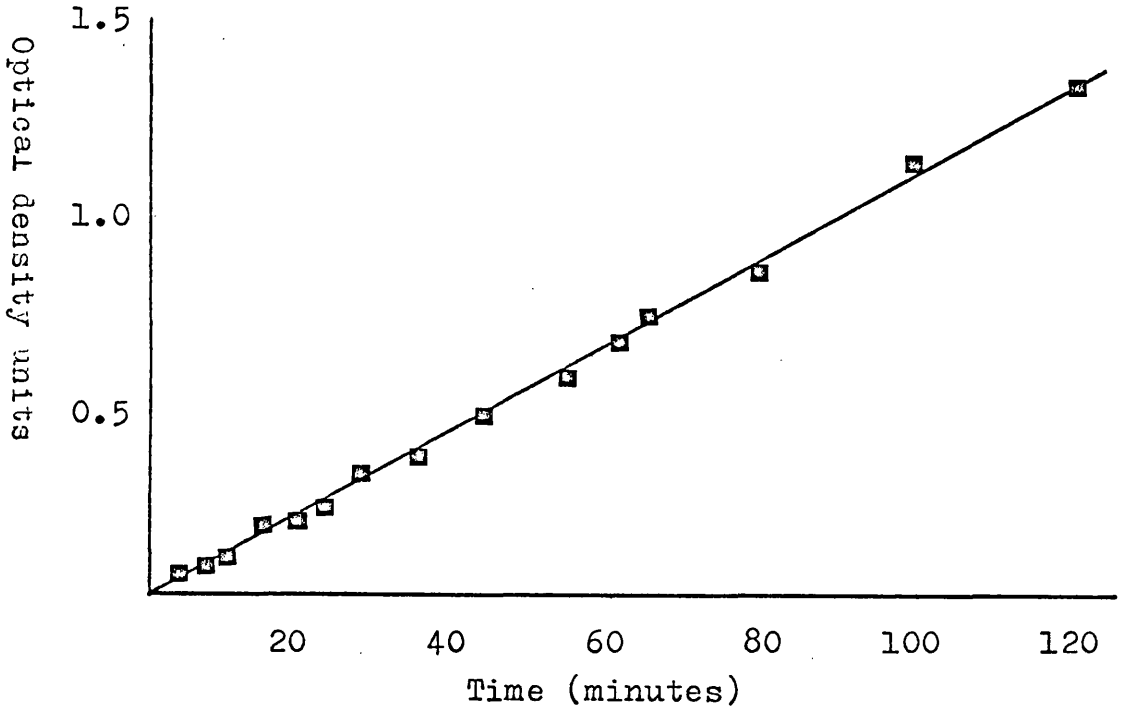


Fig 6. Relationship of P.T.E. Concentration with Activity

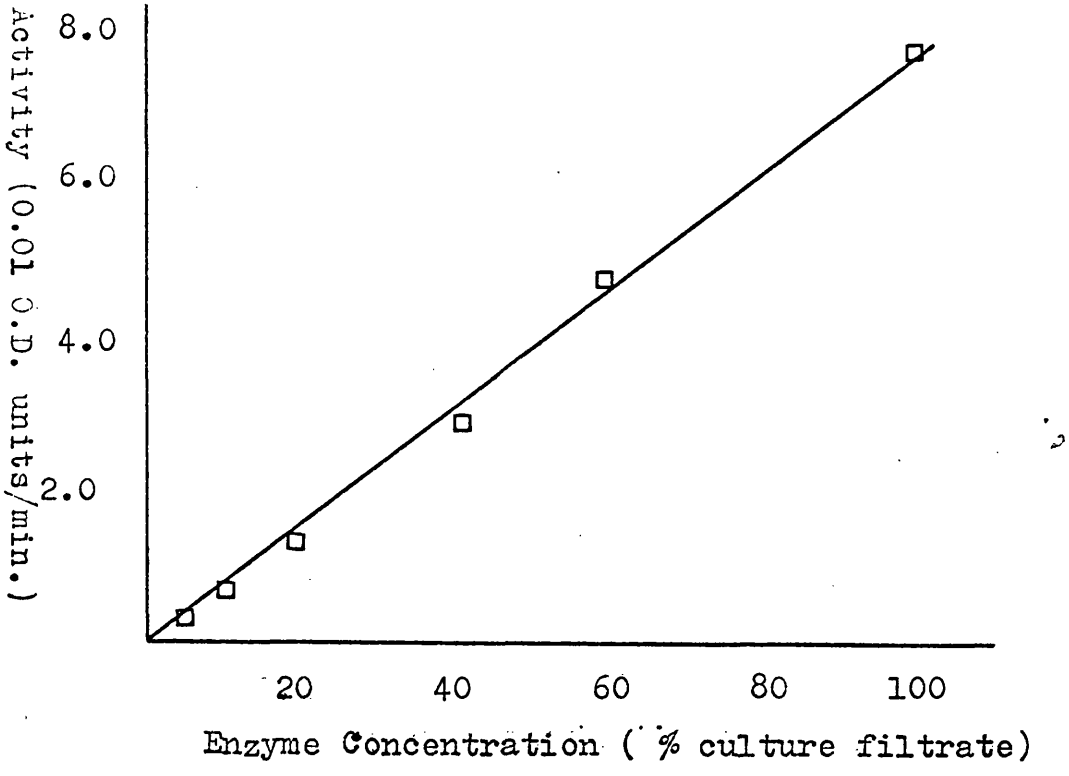


Fig 7. Relationship between Galacturonic Acid Concentration (Reducing Group) and Milli Equivalents of Iodine Reduced.

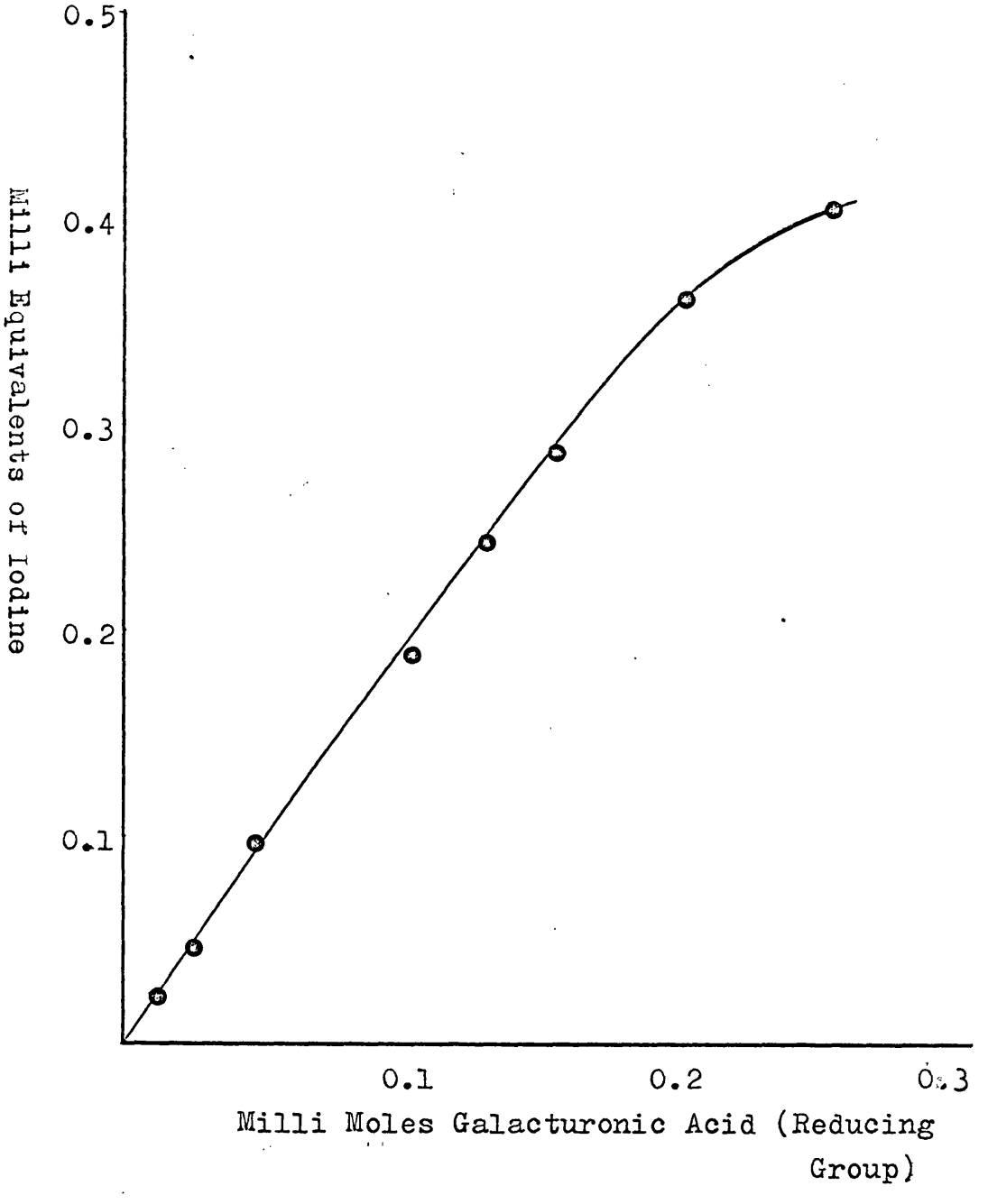


Fig 8. Release of Reducing Groups From Pectic Acid by Carterzyme.

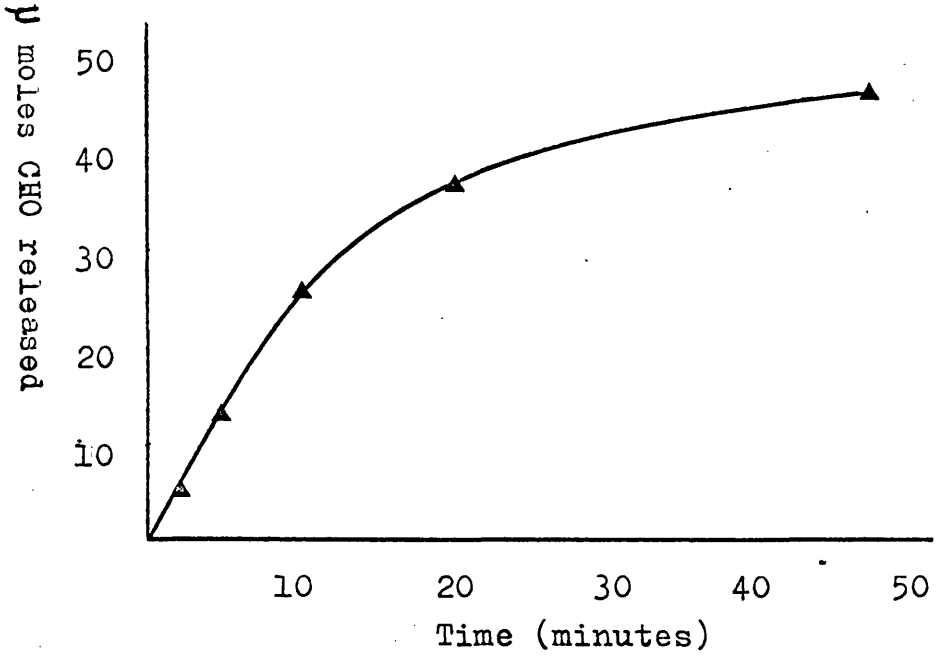
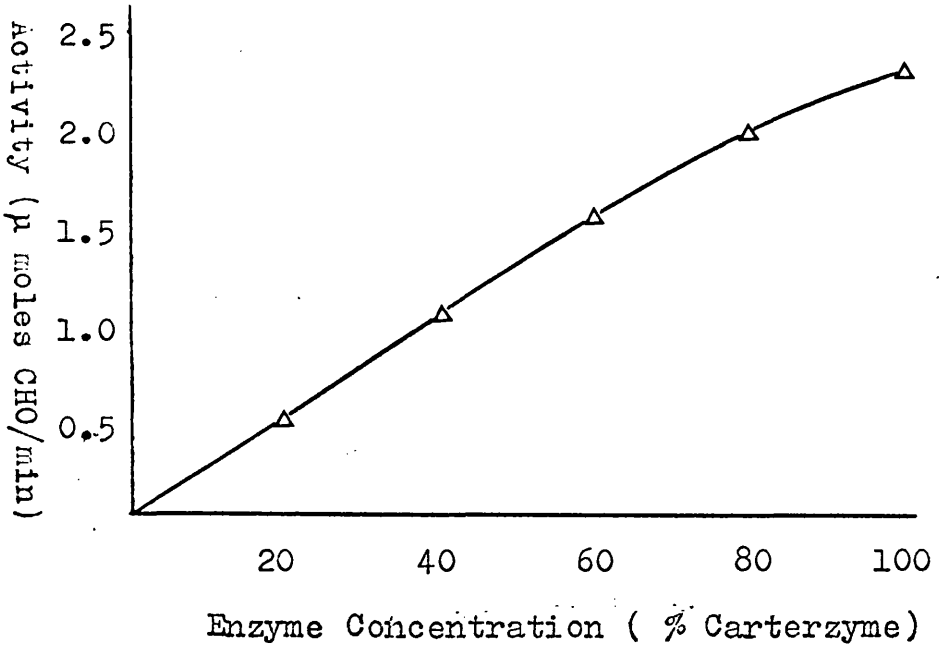


Fig 9. Relationship between Concentration and Reducing Group Liberating Activity of Carterzyme.



against the enzyme concentration as shown in Figs 12 and 13. Percentage decrease in viscosity was thus shown to be directly proportional to enzyme concentration, over the range 0 - 20% decrease in viscosity for pectin and 0 - 10% decrease in viscosity for pectic acid. In this experiment Penicillium digitatum culture filtrate was used to reduce the viscosity of pectin and Carterzyme was used with pectic acid. The reason for such a choice of enzyme-substrate systems was that, while P.digitatum culture filtrate contained an enzyme directly effecting the viscosity of pectin (P.T.E.), Carterzyme had an enzyme acting directly on pectic acid (endo-P.G.). Neither enzyme preparation affected the viscosity of the other substrate.

b. Determination of Methoxyl Content of Pectic Substances used as Substrates.

As has been mentioned earlier, one of the criteria used in classifying pectic enzymes is the nature of the substrate attacked, with respect to its degree of methylation. As no information was available on the methoxyl content of the commercial preparations used as substrates in the present work, it was decided to attempt to determine these values, in order to define more specifically the enzymes investigated. The method used to determine such methylation values was developed by Nanji and Norman (1926) and has been described earlier.

The substrates examined by this method were the following:-

1. Kodak Citrus Pectin (Prac.) - Eastman Organic Chemicals P2569

2. Sodium Polypectate, prepared from Citrus Pectin by the Method of Jermyn and Tom kins (1950).

Fig 10. The Reduction in Viscosity of a 0.5% Pectin Solution by Penicillium digitatum Culture Filtrate over 1 Hour.

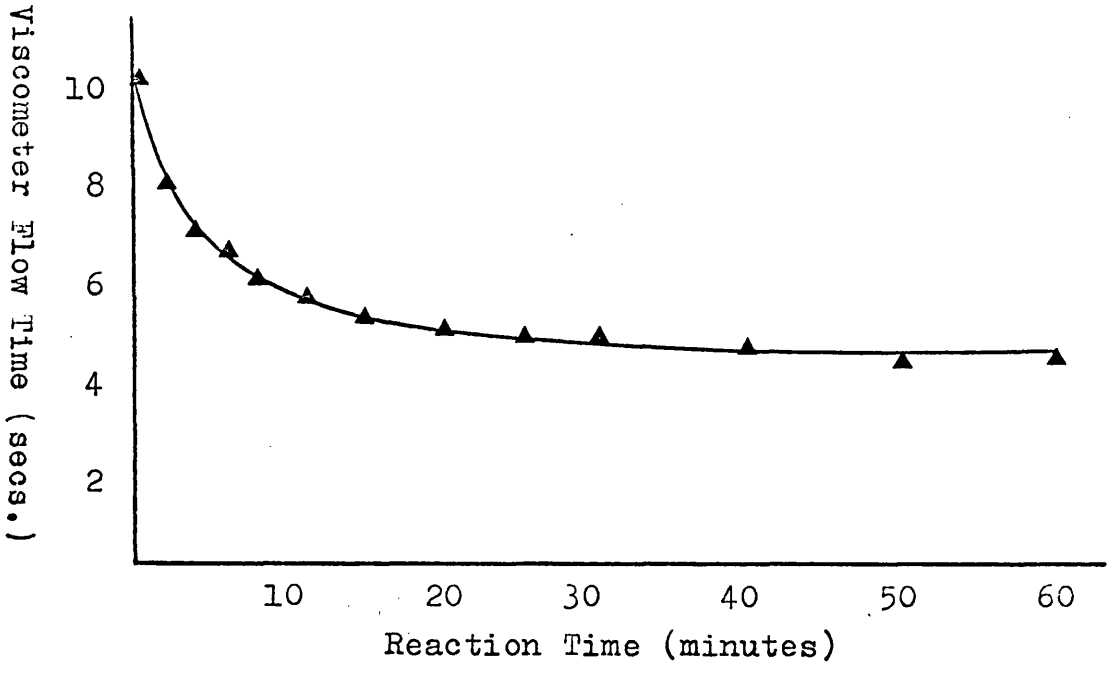


Fig 11. The Reduction in Viscosity of a 2.0% Pectic Acid Solution by Carterzyme over 1 Hour.

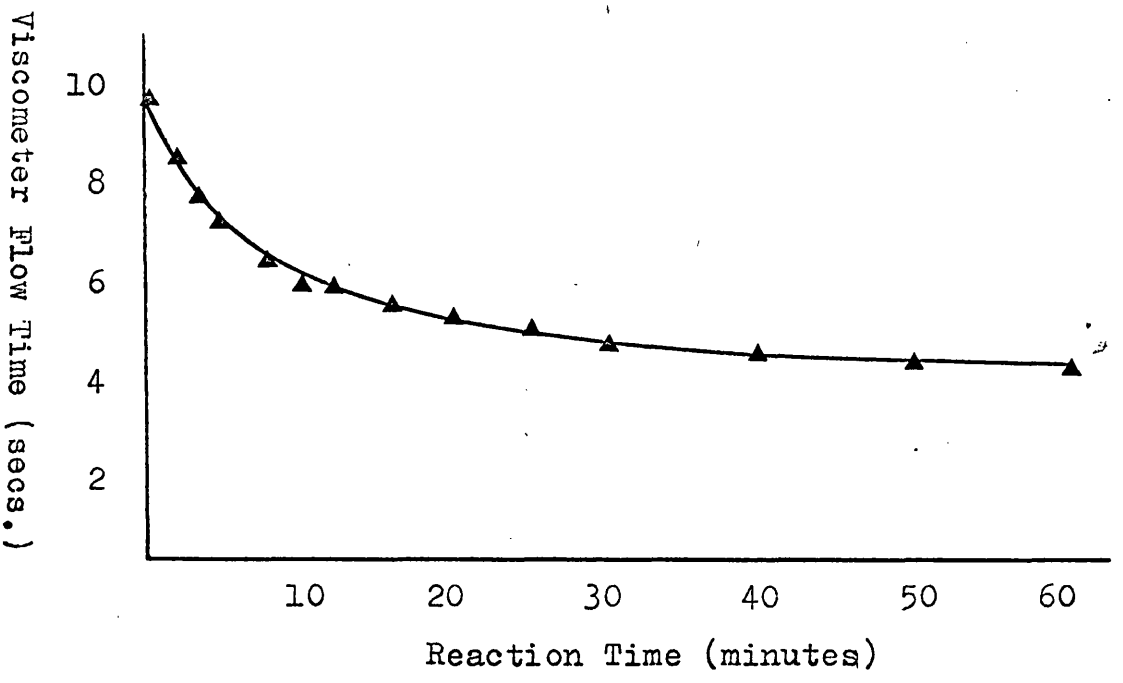


Fig 12. Relationship between Percentage Decrease in Viscosity of a 0.5% Pectin Solution and Enzyme Concentration.

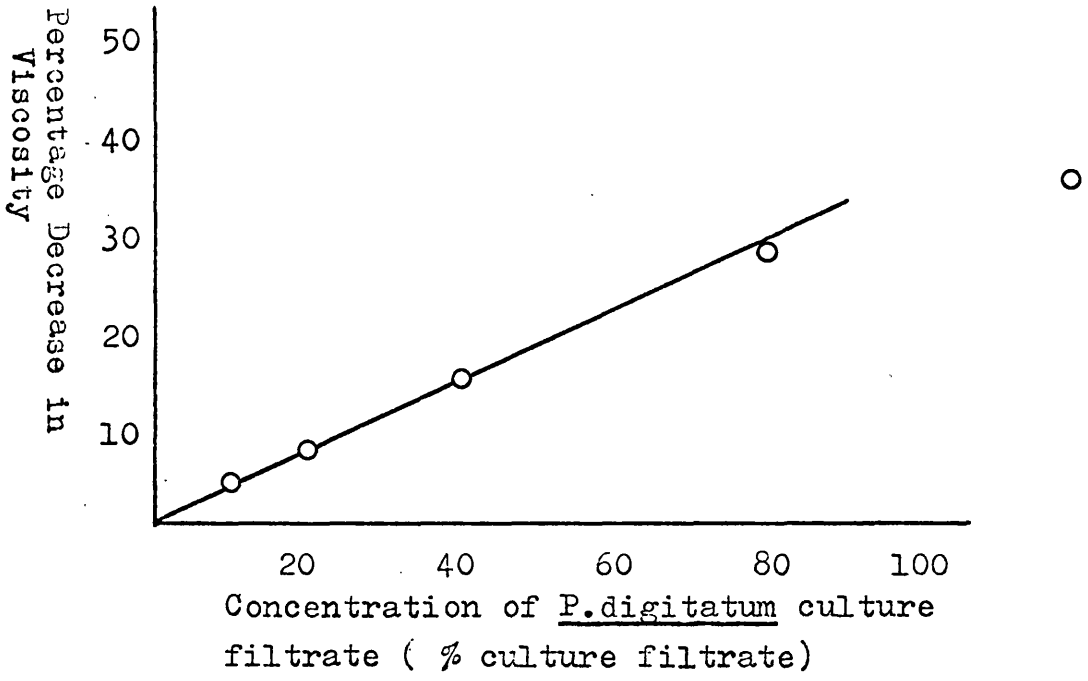
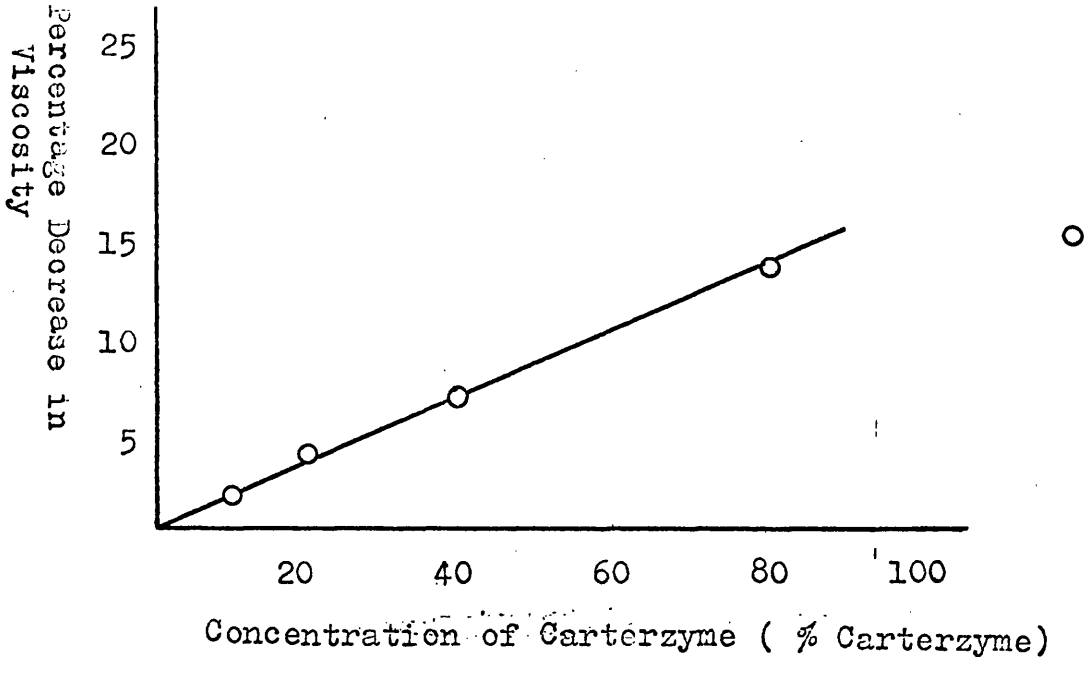


Fig 13. Relationship between Percentage Decrease in Viscosity of a 2.0% Pectic Acid Solution and Enzyme Concentration.



3. Kodak Polygalacturonic Acid - Eastman Organic Chemicals P4429.

4. Pectic Acid - K.and K. Labs. Inc. 17697

After calibration with methanol, the estimation was applied to distillates of each of these substances. The results of such an analysis are shown in table II, percentage methoxyl being calculated as:-

$$\frac{\text{the wt. of methanol}}{\text{the wt. of substance}} \times 100$$

Table II. Methoxyl Content of Pectic Substances

Substance Analysed.	Percentage Methoxyl.	Fraction of COOH groups Methylated.
1.Pectin	6.5	0.42
2.Sodium Polypectate	2.4	0.16
3.Polygalact-uronic acid	0.7	0.05
4.Pectic acid	1.2	0.08

The fraction of carboxyl groups in a molecule that are methylated, is calculated on the basis that fully methylated pectin would contain 15.4% methoxyl groups. This figure is reached theoretically from the molecular weights of galacturonic acid and methanol as follows:-

M.W. Galacturonic acid = 194.6

M.W. Methanol = 32.0

M.W. Methylated galacturonic acid = 208.6

It can be seen there fore that fully methylated pectin would have a methanol : pectin ratio of 32 : 208 or 0.154, ie fully methylated pectin would contain 15.4% methanol. Thus a sample of pectin containing x% methoxyl would have x/15.4 of its carboxyl groups methylated.

c. Preliminary Comparison of Enzyme Production by Penicillium digitatum and Penicillium notatum

A preliminary investigation of physiological aspects of Penicillium notatum, which will not invade citrus fruits was made and compared with certain aspects of the physiology of Penicillium digitatum. It was found that if an orange was sliced in half and seeded with P.notatum spores, this organism grew readily over the cut surface of the fruit. However it did not penetrate the tissue, nor did it cause any softening of the fruit as is apparent when an orange is infected with P.digitatum. From this experiment a number of conclusions could be drawn regarding the differential abilities of P.digitatum and P.notatum to parasitise oranges. The inability of P.notatum to attack oranges was not therefore, as had been suggested by Kirby (1964, unpublished), due to its inhibition at the pH of citrus tissue, its inhibition by substances present in the citrus tissue, or its inability to utilize citrus tissue as a suitable nutrient source. It appeared that the most significant difference between these two moulds, was the inability of Pnotatum , even when it had grown on cut fruit, to penetrate beyond the outer layer of damaged cells. This was coupled with the fact that it produced no softening of the fruit. It was therefore concluded that P.digitatum produced on oranges, a factor capable of causing

the tissue to soften and eventually to disintegrate; and it seemed likely that this factor enabled the organism to spread throughout the orange rind tissue.

When liquid cultures of both organisms were grown in malt extract medium, it was found that the culture filtrate of Penicillium digitatum caused softening and eventually disintegration (maceration) of orange rind discs placed in it, whereas Penicillium notatum filtrate had no such action. If the P.digitatum filtrate was boiled, then its ability to macerate orange tissue was lost, suggesting that the factor responsible for this activity was enzymic in nature.

Filtrates of both organisms were allowed to act on finely cut orange rind and the sugars in the resulting digests were separated by thin layer chromatography. The Penicillium digitatum digest was found to contain arabinose and a trace of galacturonic acid, but the Penicillium notatum digest contained only a trace of galacturonic acid. Both digests contained glucose. These results suggested that P.digitatum produced an arabanase not produced by P.notatum, but that both organisms synthesised an enzyme capable of producing galacturonic acid from orange rind. It was thought likely that this was an exo-P.G., which removes galacturonic acid units from the end of pectin chains, after this has been demethylated by P.M.E.. Confirmation that both organisms produced a P.M.E. and a P.G. was obtained using the cup plate assay techniques. Culture filtrates were examined for enzymes of the transeliminase type, by observing the change in U.V. absorption at 235 nm of a clarified citrus pectin solution. P.T.E. was detected in P.digitatum culture filtrate, but not in that of P.notatum. These results indicated that P.T.E. and arabanase might be

more important in maceration and pathogenicity than exo-P.G. and P.M.E. Such results are in agreement with the work of McClendon (1964) and Bateman and Millar (1966).

d. Investigation of Pectic Enzyme Production by Penicillium digitatum, Penicillium italicum and Aspergillus fonsecaeus on a Variety of Media.

Preliminary work has indicated that arabanase and P.T.E. might be involved in the pathogenicity of Penicillium digitatum on citrus fruits. It was therefore decided to examine in greater detail, the array of pectic enzymes produced by this organism on different media and to compare these with the enzymes produced by Penicillium italicum and Aspergillus fonsecaeus on the same media. The object of such an investigation was twofold. Firstly it was hoped to gain some additional information on the association of particular enzymes with macerating activity and secondly the investigation was designed to show to what extent the three citrus pathogens showed any similarity in the production of pectic enzymes.

The three media used in this investigation were:-

1. 3% malt extract + 0.5% mycological peptone
2. 3% malt extract + 0.5% mycological peptone + 0.5% pectin.
3. Bran.

Both surface and submerged cultures were grown in the liquid media, in 250ml conical flasks at 25°C. The bran cultures were also grown in 250ml flasks at 25°C. When the cultures were harvested, the mycelium of the liquid cultures was dried and weighed and the amount of extracellular protein in each filtrate was

measured. The enzyme assays carried out were those for P.T.E., arabanase, enzymes reducing the viscosity of pectin and pectic acid, enzymes liberating reducing groups from pectic acid, P.M.E. and macerating activity.

Penicillium digitatum cultures were inoculated with 1.25×10^8 spores per flask. Submerged cultures were grown for 48 and 72 hours, surface cultures for 144 and 168 hours and bran cultures were grown for 120 and 144 hours. Frequently difficulty was encountered in obtaining a continuous mycelial mat in the case of surface cultures, although this occurred more easily on the medium containing pectin than on the malt extract alone. The results of such an investigation are shown in table III.

Cultures of Penicillium italicum were inoculated with 1.5×10^8 spores per flask. Submerged cultures were harvested after 48 and 72 hours growth, surface cultures after 120 and 148 hours and bran cultures after 72 and 96 hours. This organism grew considerably faster on bran than did Penicillium digitatum but the same difficulty in obtaining a continuous mycelial mat in surface cultures was encountered. The results of this experiment are shown in table IV.

Cultures of Aspergillus fonsecaeus were inoculated with 1.25×10^8 spores per flask. This organism grew both quickly and profusely, especially in submerged cultures and these were therefore harvested after 24 and 48 hours growth. Surface cultures were harvested after 120 and 144 hours and bran cultures after 72 and 96 hours. The effect of growth conditions on enzyme production by A.fonsecaeus are shown in table V. Although growth, as measured by dry weight of mycelium, was from two to three times greater than with the two Penicillia, protein production by

Table III. Enzymes Produced by Penicillium digitatum under a Variety of Growth Conditions.

Growth Conditions.	Dry Wt. of Mycelium(g).	Protein (mg/ml)	P.G. Activity (μ moles reducing group/min)	P.M.E. (presence in plate assay)	P.T.E. Activity. (0.01 OD. units/min.)	Viscosity Reducing Activity (% reduction in viscosity/min.)		Arabanase Activity (ug arabinose/min)	Maceration (degree of softening)	
						Pectin	Pectic Acid		2hr.	24hr.
1. Sb M 48.	0.224	2.8	0	-	4.07	17.1	0	5.7	++	++++
2. Sb M 72.	0.197	2.7	0	-	4.17	17.1	0	10.0	++	++++
3. Sb MP 48.	0.274	2.1	0	-	2.04	13.1	0	22.4	+	+++
4. Sb MP 72	0.252	2.0	0	-	1.43	11.2	0	11.6	+	+++
5. Sr M 144	0.432	2.6	0	+	2.29	13.1	0	10.5	+	+++
6. Sr M 168	0.443	2.3	0	+	3.00	15.0	0	14.3	+	+++
7. Sr MP 144	0.339	2.2	0	+	1.45	9.40	0	16.4	+	+++
8. Sr MP 168	0.415	2.1	0	+	1.59	10.2	0	25.4	+	+++
9. B 120		2.1	0.2	+	4.93	24.2	2.2	45.7	++	++++
10. B 144		1.8	0.13	+	3.02	12.9	2.9	44.3	++	++++

Key. Sb.= Submerged culture. Sr.= Surface culture.
M = Malt medium. MP = Malt + Pectin medium.
B = Bran. Figure = growth time in hours.

Table IV. Enzymes Produced by Penicillium italicum under a Variety of Conditions.

Growth Conditions.	Dry Wt. of Mycelium(g)	Protein (mg/ml)	P.G. Activity (u moles reducing group/min)	P.M.E. (presence in plate assay)	P.T.E. Activity (0.01 OD. units/min.)	Viscosity Reducing Activity (% reduction in viscosity/min.)		Arabanase Activity (ug arabinose/min)	Maceration (degree of softening)	
						Pectin	Pectic Acid		2hr.	24hr.
1. Sb M 48	0.307	2.4	0	+	0.11	0	0	2.0	±	±
2. Sb M 72	0.296	2.6	0	+	0.08	0	0	2.0	±	±
3. Sb MP 48	0.412	2.0	0	+	0.14	0	0	4.5	±	±
4. Sb MP 72	0.398	2.0	0	+	0.27	0	0	3.8	±	+
5. Sr M 120	0.142	1.8	0	+	0.71	10.0	0	9.4	+	++
6. Sr M 148	0.193	1.7	0	+	0.83	8.0	0	8.6	+	++
7. Sr MP 120	0.158	1.6	0	+	0.11	0	0	13.9	±	-
8. Sr MP 148	0.148	1.6	0	+	0.16	0	0	14.2	±	-
9. B 72		1.9	0.10	+	3.11	17.0	0	9.5	++	+++
10. B 96		1.9	0.13	+	3.06	17.0	0	9.7	++	+++

Key. Sb = Submerged culture. Sr = Surface culture
M = Malt medium MP = Malt + Pectin medium
B = Bran Figure = growth time in hours

Table V. Enzymes Produced by Aspergillus fonsecaeus under a Variety of Conditions.

Growth Conditions.	Dry Wt. of Mycelium(g)	Protein (mg/ml)	P.G. Activity (u moles reducing group/min)	P.M.E. (Presence in plate assay)	P.T.E. Activity (0.01 OD. units/min)	Viscosity Reducing Activity (% reduction in viscosity/min.)		Arabanase Activity (ug arabinose/min.)	Maceration (degree of softening)	
						Pectin	Pectic Acid		2hr.	24hr.
1. Sb M 24	0.67	1.77	0	+	0.17	1.4	0	2.0	-	±
2. Sb M 48	1.01	1.10	0	+	0	0	0	2.9	-	±
3. Sb MP 24	0.58	1.38	0	+	0	0	0	4.0	-	±
4. Sb MP 48	0.82	0.93	0	+	0	0	0	6.1	-	±
5. Sr M 120	0.89	1.80	0	+	0	0	0	4.7	±	++
6. Sr M 144	0.89	1.68	0	+	0	0	0	4.5	+	++
7. Sr MP120	0.65	1.23	0	+	0	0	0	4.7	+	+++
8. Sr MP148	0.73	1.24	0	+	0	0	0	5.1	+	++
9. B 72		3.40	0.15	+	0	2.8	2.0	40.5	±	+++
10. B 96		3.29	0.10	+	0	3.2	2.3	33.8	±	+++

Key. Sb = Submerged culture. Sr = Surface culture.
M = Malt medium MP = Malt + Pectin med:
B = Bran Figure = growth time in hours

A.fonsecaeus was slightly less than with these fungi in the liquid media. Protein production by A.fonsecaeus on bran was however significantly higher than that of Penicillium digitatum and Penicillium italicum. Enzyme production was generally less by A.fonsecaeus than by P.digitatum and P.italicum. P.M.E. was produced by all three fungi under all growth conditions except the submerged cultures of P.digitatum. Exo-P.G. as measured by the liberation of reducing groups from pectic acid was produced only in bran cultures of each organism. Arabanase was produced by all three organisms, though in greatest quantity by P.digitatum. Bran appeared to be the most satisfactory substrate for the production of this enzyme, except in the case of P.italicum, when liquid surface cultures were equally satisfactory.

P.T.E. was produced in all media by Penicillium digitatum and Penicillium italicum , not to any great extent by the latter, except in bran. P.T.E. was not detected in cultures of Aspergillus fonsecaeus, apart from a trace in young submerged cultures, without pectin. Pectin appeared to have an inhibitory effect on the production of P.T.E. (c.f. tables III.and IV.), a phenomenon that does not seem to have been reported previously. The reason for this is at present unknown, although it is considered that it might be explained in terms of either enzyme substrate absorption, or product inhibition. In either case P.T.E. would be produced by the organism in question, but would then start to break down the pectin in the medium. In doing this,it is possible that some of the enzyme might become bound to the pectin, so that it would not show any activity in the assay. Alternatively the enzyme might be partially inactivated by the products of the pectin breakdown, and again the organism would appear

to have produced less P.T.E. in the pectin medium. Product inhibition is by no means an unknown phenomenon, although it must be admitted that no evidence has yet been found to verify either of these explanations.

The activity of the enzyme(s) causing a reduction in viscosity of pectin, followed the activity of P.T.E. in most cases, although where P.T.E. activity was very low, as in the liquid cultures of P.italicum, no viscosity reducing activity on pectin was recorded. Enzymes reducing the viscosity of pectic acid were only produced on cultures grown on bran, as were enzymes causing the liberation of reducing groups from pectic acid. It may be therefore that there is an association between these activities.

Macerating activity was produced under the same conditions as P.T.E. by Penicillium digitatum and Penicillium italicum. This was not the case however in Aspergillus fonsecaeus, where macerating activity was present in the surface and bran cultures, but P.T.E. activity was not detected. The indication is therefore, that P.T.E. may be responsible for the tissue maceration caused by P.digitatum and P.italicum, but that this is most unlikely to be the case with A.fonsecaeus. This view was investigated further by applying enzyme separation proceedures to filtrates of the three organisms, in an attempt to purify the enzyme(s) responsible for the maceration process. Growth conditions chosen for the production of filtrates for separation, were those which stimulated the greatest production of macerating factor and P.T.E.

e. Separation and Purification of Enzymes from Culture Filtrates of Penicillium digitatum, Penicillium italicum and Aspergillus fonsecaeus.

1. Penicillium digitatum. Submerged cultures of Penicillium digitatum were grown in a malt extract medium and harvested after 48 hours. Four litres of culture filtrate was freeze dried and re-dissolved in water to 1 litre. This concentrated filtrate was fractionated by an ammonium sulphate precipitation, the results of which are shown in table VI. Solutions of precipitates from this fractionation were made in 100ml, and that solution prepared from the precipitate formed at 0.5 $(NH_4)_2SO_4$ saturation, which contained the greatest P.T.E. activity, was subjected to a second fractionation with ammonium sulphate. Solutions of the precipitates from the second fractionation were made in 15ml and were assayed for enzyme activity, as shown in table VII. The solutions of precipitates formed at 0.4 and 0.5 $(NH_4)_2SO_4$ saturation in the second fractionation, were passed through a column of Sephadex G25 to desalt them, and then through a column of ECTEOLA cellulose to effect a separation of P.T.E. and arabanase. The enzymes were eluted by water and stepwise additions of increasing concentrations of sodium chloride, as shown in Fig 14.

The specific activity of P.T.E. was increased from 18.8 units/mg protein in the culture filtrate, to 4852.0 units/mg protein in the purest fraction (fraction 5) from the ECTEOLA cellulose column, giving an overall 258 fold increase in specific activity for the purification process. Similarly the specific activity of the arabanase was increased from 8.1 units/mg protein in the culture filtrate, to 495 units/mg protein in fraction 21 from the ECTEOLA cellulose, giving an overall 61 fold increase for the purification process.

Table VI. First Ammonium Sulphate Fractionation of Penicillium digitatum Culture Filtrate

Fraction	P.T.E.		Arabanase		Maceration
	Activity (0.01 O.D. units/min.)	Specific Activity (units activity per mg protein)	Activity (ug arabinose/min)	Specific Activity (units activity per mg protein)	(Degree of Softening)
Culture Filtrate	6.2	18.8	26.7	8.1	+
Conc. Culture Filtrate	27.4	20.8	136.7	10.4	+++
$(NH_4)_2SO_4$ Sat.					
0.2	0	-	5.2	2.3	-
0.4	30.0	97.4	346.7	112.6	+++
0.5	206.0	647.8	640.0	201.6	+++ *
0.6	30.2	95.0	110.7	34.8	+++
0.8	2.4	4.3	43.3	7.8	+
1.0	0	-	10.0	2.8	-
Residue (material not ppted. by sat. $(NH_4)_2SO_4$	0	-	13.0		-

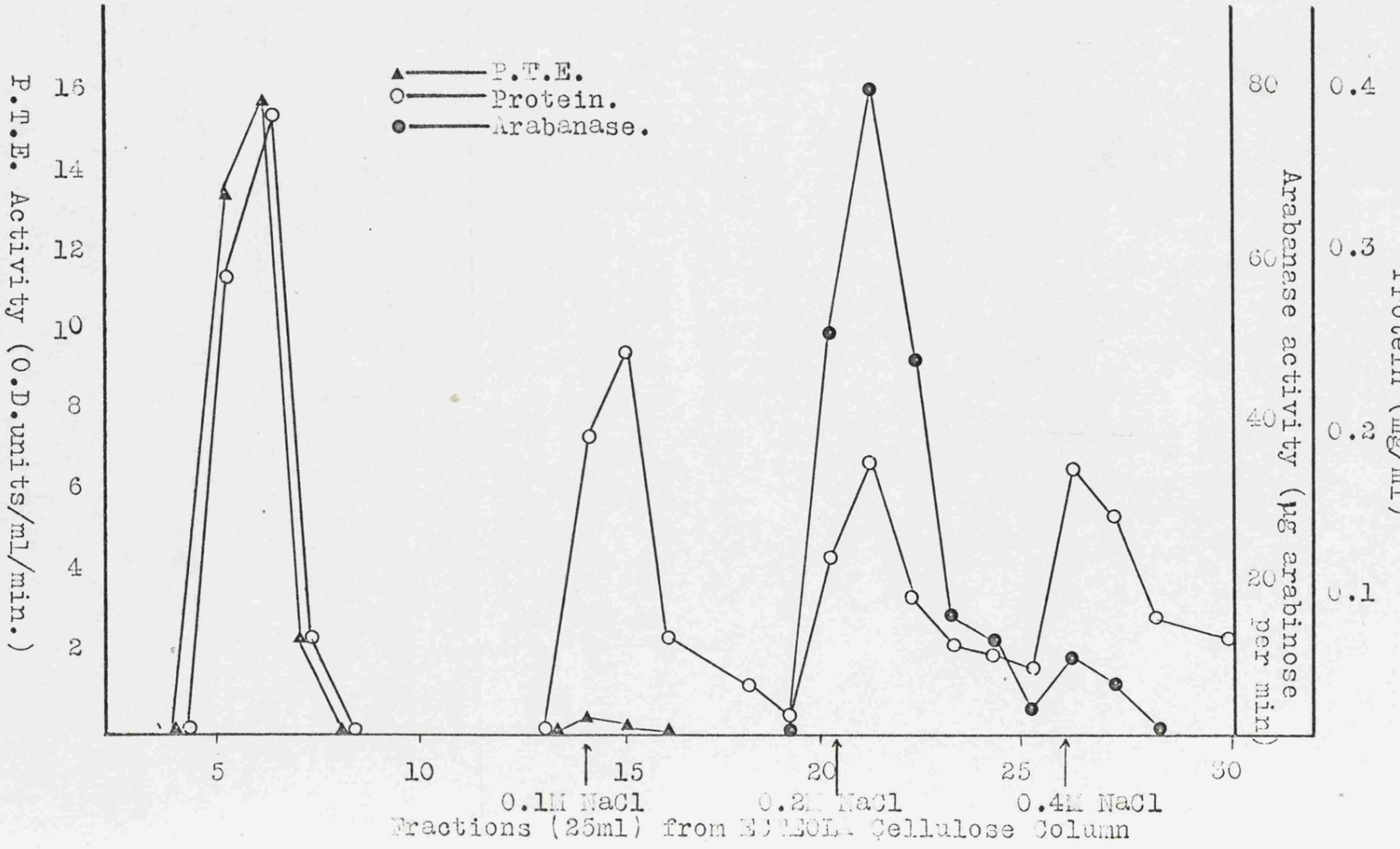
(* = 1/10th. dilution, otherwise undiluted fractions used)

Table VII. Second Ammonium Sulphate Fractionation using the Precipitate Formed at 0.5 $(NH_4)_2SO_4$ Saturation in Table VI.

Fraction	P.T.E.		Arabanase		Maceration (Degree of Softening)
	Activity (0.01 OD. units/ min.)	Specific Activity (units activity per mg protein)	Activity (µg arabinose/ min.)	Specific Activity (units activity per mg protein)	
0.5 sat. $(NH_4)_2SO_4$ ppt. from tableVI.	2266.0	647.8	640.0	201.6	+++ *
Saturation.					
0.2	54.0	135.0	5.2		+
0.4	4120.0	710.0	1174.0	202.4	+++ **
0.5	2618.0	808.0	55.0	17.1	+++ *
0.6	82.0	49.4	0	-	+
0.8	0	-	0	-	-
1.0	0	-	0	-	-
Residue (material not ppted. by sat. $(NH_4)_2SO_4$	0	-	0	-	-

(* = 1/10th dilution, ** = 1/20th dilution, otherwise undiluted fractions used)

Fig. 14. The Separation of P.T.E. and Arabanase from Penicillium digitatum by Column Chromatography on ECTEOLA Cellulose.



Arabanase activity was separated from P.T.E. by this process, and fractions containing only arabanase did not produce maceration. It was not found possible however, to separate P.T.E. and macerating activity. This was the first direct evidence obtained in the present work, indicating that P.T.E. rather than arabanase was responsible for maceration.

2. Penicillium italicum. Penicillium italicum bran cultures were set up in 1 litre conical flasks, using 50g of bran per flask. The cultures were harvested by extraction with water, after 72 hours, as described previously, and the 4 litres of 'filtrate' so obtained were freeze dried and reconstituted to 1 litre. To this concentrated bran filtrate was added ammonium sulphate as with the Penicillium digitatum culture filtrate, and the precipitate formed at each saturation was dissolved in water and assayed for enzyme activity. The results of such a fractionation are shown in table VIII.

The solution of the precipitate formed at 0.5 $(NH_4)_2SO_4$ saturation was subjected to a further ammonium sulphate fractionation. The precipitates so formed were then dissolved in 15ml of water and assayed for enzyme activity as shown in table IX. The solution of that precipitate formed at 0.5 $(NH_4)_2SO_4$ saturation in table IX was passed through a column of Sephadex G25 to remove salts and was then subjected to column chromatography on ECTEOLA cellulose. Separation of P.T.E. and arabanase was effected using a stepwise elution with sodium chloride, as shown in Fig 15. The P.T.E. from Penicillium italicum was denatured to a greater extent than that from Penicillium digitatum during the purification process.

Table VIII. First Ammonium Sulphate Fractionation of Penicillium italicum Bran Filtrate.

Fraction	P.T.E.		Arabanase		P.G.		P.M.E.	Maceration
	Activity (0.01 OD units/min)	Specific Activity (units activity/mg protein)	Activity (ug arabinose/min)	Specific Activity (units activity/mg protein)	Activity (u moles reducing group/min)	Specific Activity (units activity/mg protein)	Presence in plate Assay.	Degree of Softening
Bran Filtrate	7.1	15.0	26.7	6.3	0.32	0.15	+	+
Conc.Filtrate	15.4	16.9	61.7	7.6	0.64	0.16	+	++
$(NH_4)_2SO_4$ Saturation								
0.2	12.0	6.6	0	-	0	-	-	++
0.4	24.5	49.7	126.7	25.7	0	-	-	++++
0.5	76.0	253.3	126.7	42.2	0	-	-	++*
0.6	23.9	52.5	20.0	4.0	0	-	-	+++
0.8	2.2	1.7	90.0	6.9	1.96	0.30	+	+
1.0	0.2	0.3	86.7	14.4	1.09	0.36	+	±
Residue (material not precipitated by sat. $(NH_4)_2SO_4$)	0	-	16.7	4.9	0	-	+	-

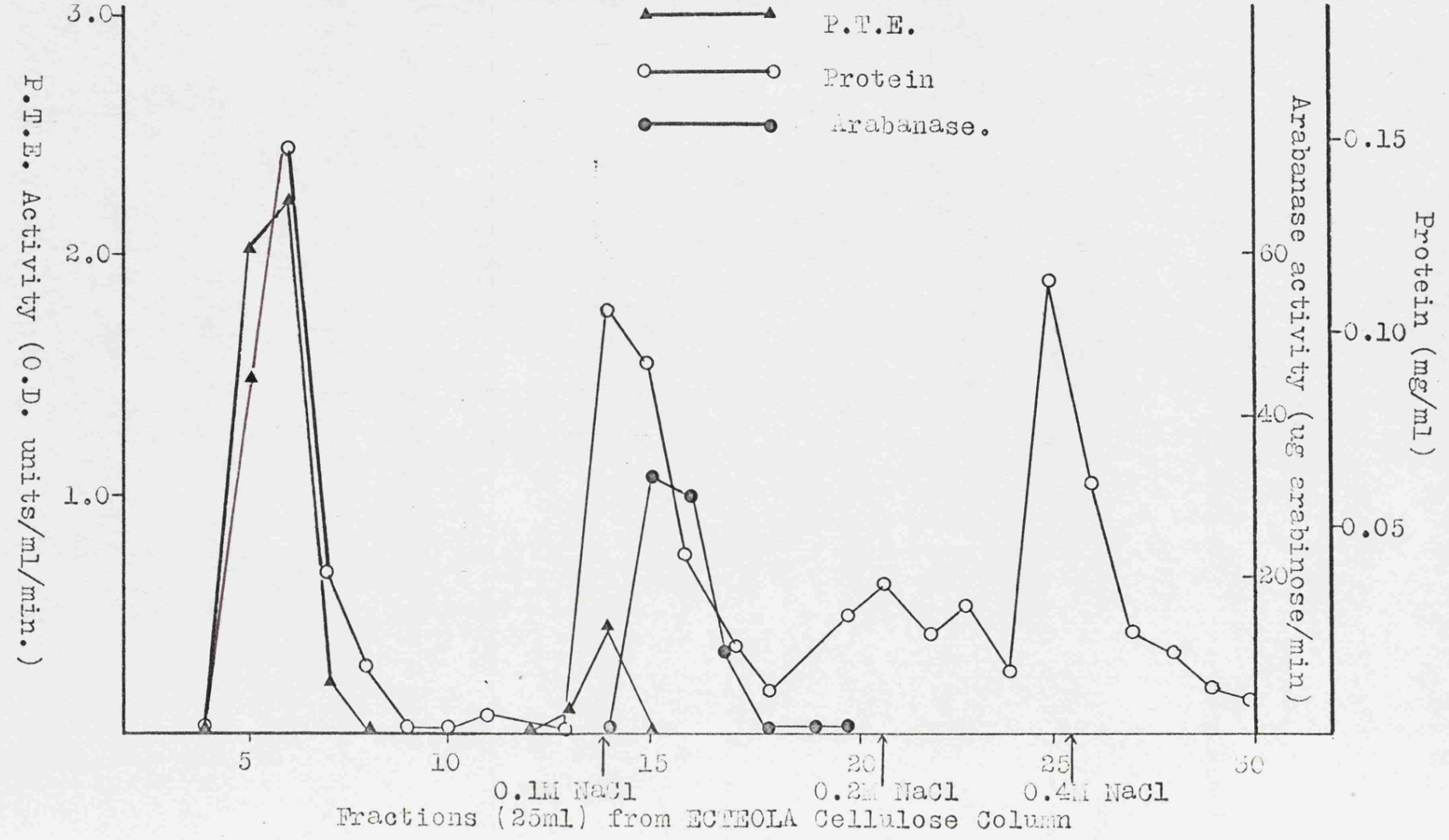
* = diluted 1/4 in the maceration assay, otherwise undiluted fractions used.

Table IX. Second Ammonium Sulphate Fractionation using the Precipitate Formed at 0.5 $(NH_4)_2SO_4$ Saturation in Table VIII. (Penicillium italicum)

Fraction	P.T.E.		Arabanase		Maceration
	Activity (0.01 OD. units per min)	Specific Activity (units per mg protein)	Activity (ug arabinose per min)	Specific Activity (units activity per mg protein)	(degree of softening)
0.5 sat. $(NH_4)_2SO_4$ ppt. from table VIII.	71.2	250.0	113.0	40.0	++++
Saturation.					
0.2	3.1	67.4	0	-	±
0.4	77.0	315.6	477.0	195.5	++++
0.5	218.6	888.6	247.0	100.4	++ *
0.6	35.8	303.4	8.0	6.8	+++
0.8	4.0	26.1	8.0	5.4	+
1.0	1.8	24.0	4.0	4.8	±
Residue (material not ppted. by sat. $(NH_4)_2SO_4$	0	-	10.0	-	-

(* = 1/10th. dilution otherwise undiluted fractions used.)

Fig. 15. The Separation of P.T.E. and Arabanase from Penicillium italicum by Column Chromatography on ECTEOLA Cellulose.



The specific activity of the P.T.E. was increased from 15.0 units/mg protein in the Penicillium italicum bran filtrate to 2174.0 units/mg protein in the purest fraction (fraction 5) from the ECTEOLA cellulose column, giving an overall increase in specific activity of 145 times. Similarly the specific activity of the arabanase was increased from 6.3 in the bran filtrate to 697.0 in fraction 16 from the ECTEOLA cellulose, giving an overall increase in specific activity of 110 times. As with Penicillium digitatum this procedure did not effect any separation of P.T.E. from macerating activity.

The precipitate formed at 0.8 $(NH_4)_2SO_4$ saturation in table VIII, appeared to have a higher macerating activity than could be accounted for by its P.T.E. content. It was therefore decided to attempt a further purification of this fraction, in order to determine the nature of other possible macerating enzymes. An ammonium sulphate fractionation was thus applied to this fraction and the solutions of the precipitates formed were assayed for enzyme activity. The results are shown in table X. From them it was clear that maceration could be produced by factors other than P.T.E. in Penicillium italicum bran filtrate. The indications were that this might be accounted for by a P.G. probably in conjunction with P.M.E. However attempts to further purify the P.G. fraction (fraction 0.8., table VIII) both by column chromatography and absorption onto pectic acid gel proved unsuccessful. The column chromatography did however, give further indication that a component other than P.T.E. and arabanase had a macerating activity, and that this component might be a P.G.

Table X. Ammonium Sulphate Fractionation of the Precipitate Formed at 0.8 $(NH_4)_2SO_4$ Saturation in Table VIII. (Penicillium italicum)

Fraction	P.T.E. (0.01 OD. units per min.)	Reducing Group Liberation (μ moles CHO per min.)	Arabanase (μg arabinose per min.)	P.M.E. (presence in plate assay)	Maceration (degree of softening)
0.8sat. $(NH_4)_2SO_4$ ppt. from table VIII.	2.2	1.96	9.0	+	++
Saturation.					
0.2	0	0.26	4.13	-	±
0.4	0	0.19	3.33	±	±
0.5	0.9	0.19	2.33	±	±
0.6	7.6	0.96	4.50	+	+
0.8	2.3	3.40	80.00	+	+
1.0	0	2.38	48.30	+	+
Residue (material not ppt. by sat. $(NH_4)_2SO_4$	0	0	10.00	-	-

3. Aspergillus fonsecaeus. The organism was grown on bran, as this medium induced the best production of macerating activity. The filtrate, prepared as before, was freeze dried and made up to 1 litre in distilled water(i.e. concentrating it 4 times). This concentrated filtrate was fractionated by an ammonium sulphate precipitation, the results of which are shown in table XI.

Contrary to the results of the experiments on enzyme production in various media, a small amount of P.T.E. was detected in the bran filtrate of this organism. Such activity appeared in the same fractions, during the ammonium sulphate precipitation, as P.T.E. activity produced by the two Penicillia. The P.T.E. in these fractions appeared to be associated with part of the macerating activity produced by Aspergillus fonsecaeus. The activity of enzymes reducing the viscosity of pectin and pectic acid was also assayed in this fractionation. Viscosity reducing activity on pectin appeared in the same fractions as P.T.E., whereas activity on pectic acid was found in the same fractions as enzymes liberating reducing groups from this substrate. Arabanase activity also corresponded with maceration to some extent. An attempt to purify the P.T.E. in the 0.5 $(NH_4)_2SO_4$ saturated fraction from table XI, by cellulose ion exchange chromatography, was unsuccessful. Only a very small fraction of the P.T.E. was eluted by water in the initial fractions from the column, the majority being eluted by 0.2 M NaCl in later fractions. This suggested that the P.T.E. produced by A.fonsecaeus differed, at least in its ionic properties, from those of Penicillium digitatum and Penicillium italicum. It was clear however from table XI, that by comparison with P.digitatum and

Table XI. Ammonium Sulphate Fractionation of Aspergillus fonsecaeus Bran Filtrate.

Fraction	P.T.E. Activity (0.01 OD. units/min)	Viscosity Reducing Activity (% reduction in viscosity per minute)		P.G. Activity (u moles reducing group/min)	Arabanase Activity (ug arabinose/min)	P.M.E. Presence in Plate Assay	Maceration Degree of Softening
		Pectin	Pectic Acid				
Bran Filtrate	1.0	5.50	3.00	0.76	62.0	+	+
Conc.Filtrate	2.6	15.50	13.00	1.50	183.0	+	++
$(NH_4)_2SO_4$ Saturation							
0.2	0.2	0	0	0.20	0	-	-
0.4	1.5	7.30	1.00	0	22.0	-	+
0.5	11.1	31.60	1.00	1.10	270.0	+	++
0.6	6.0	23.70	3.00	1.50	587.0	+	++
0.8	7.0	25.50	28.00	2.10	613.0	+	++
1.0	0.5	12.30	25.00	2.30	53.0	+	++
Residue (material not precipitated by sat. $(NH_4)_2SO_4$)	0	1.30	3.00	0	23.0	+	+

P.italicum the P.T.E. produced by A.fonsecaeus under the conditions used, was insufficient to account for the maceration produced by this organism.

The viscosity reducing activity on pectic acid was found in fractions causing high maceration, pointing to a possible association of these two activities. The enzyme responsible for reducing the viscosity of pectic acid is most likely to be an endo-P.G. Maceration of plant tissues by endo-P.G.s usually involves P.M.E. (Bateman and Millar 1966), an enzyme which does in fact occur in fractions from Aspergillus fonsecaeus having endo-P.G. and macerating activity.

Although arabanase was also found in the same fractions as macerating activity, it was not considered that this played a part in maceration, as arabanase fractions have been found free of macerating activity during the fractionation of culture filtrates of Penicillium digitatum and Penicillium italicum. It is realized however, that Aspergillus fonsecaeus arabanase may differ from the arabanase produced by the Penicillia although no evidence for this has been found.

f. Comparison of the Properties of the P.T.E.s from Penicillium digitatum and Penicillium italicum

Samples of P.T.E. from Penicillium digitatum and Penicillium italicum were prepared by the fractionation procedures previously described. The protein content was measured by the Lowry method and samples of known protein content were then freeze dried. The dry weight of these samples corresponded to the protein values, previously calculated.

1. Electrophoretic Homogeneity. Samples of both transeliminases were examined for electrophoretic homogeneity by disc electrophoresis, as previously

described. 0.1ml of solution, containing about 250µg of protein were applied to the tubes, and run at 5m.a. per tube. The P.T.E.s from Penicillium digitatum and Penicillium italicum were run singly and in mixtures of equal parts of each. In every case however, only one protein band was detected on the gel, as shown in plate 1. This suggested firstly, that each trans-

Plate 1. Electrophoresis of P.T.E.s from Penicillium digitatum and Penicillium italicum.

eliminase was electrophoretically homogeneous and secondly that both enzymes had the same electrophoretic mobility. Such evidence points strongly to the fact that the enzymes from both organisms may be identical in structure. To obtain further evidence for or against

this view, it was decided to examine the effect of-reaction conditions on both P.T.E.s. The effect of reaction conditions on the macerating activity and the pectin viscosity reducing activity, still found in the purified P.T.E. preparations, was also investigated.

2. Scan of Reaction Products. The P.T.E.s from Penicillium digitatum and Penicillium italicum were allowed to react with buffered pectin at pH 5.2 for 15 minutes. After this time wavelength scans of the reaction products were carried out on a Unicam SP 800 recording spectrophotometer, using a buffered pectin solution as a blank, in order to determine the absorption peaks of these mixtures. The results are shown in Figs 16 and 17, and the peaks for both reaction mixtures appeared to be about 239 nm., rather than the reported value of 235 nm. However the latter value was so little removed from the peak obtained in the scans, that it was decided to continue to measure P.T.E. activity at this value for the sake of consistancy.

3. The Effect of Substrate Concentration on the Activity of the P.T.E.s. The effect of substrate concentration on enzyme activity varies with different enzyme substrate systems. The Michaelis constant, or the substrate concentration when half the maximum activity is reached, is a measure of the affinity of an enzyme for its substrate, and can be used to compare different enzyme substrate systems. The most usual method of obtaining the Michaelis constant (Km), is to plot $1/v$ against $1/S$, where v = the initial velocity or activity and S = the substrate concentration. Km is then taken as the reciprocal of the value where the plot bisects the negative part of the abscicca. The substrate concentration is measured in molarities for the purpose of obtaining Km. Therefore in the case of P.T.E. acting

Fig 16. Absorption of the Reaction Products of P.T.E. from Penicillium digitatum and Pectin at pH 5.2. (traced from original scan).

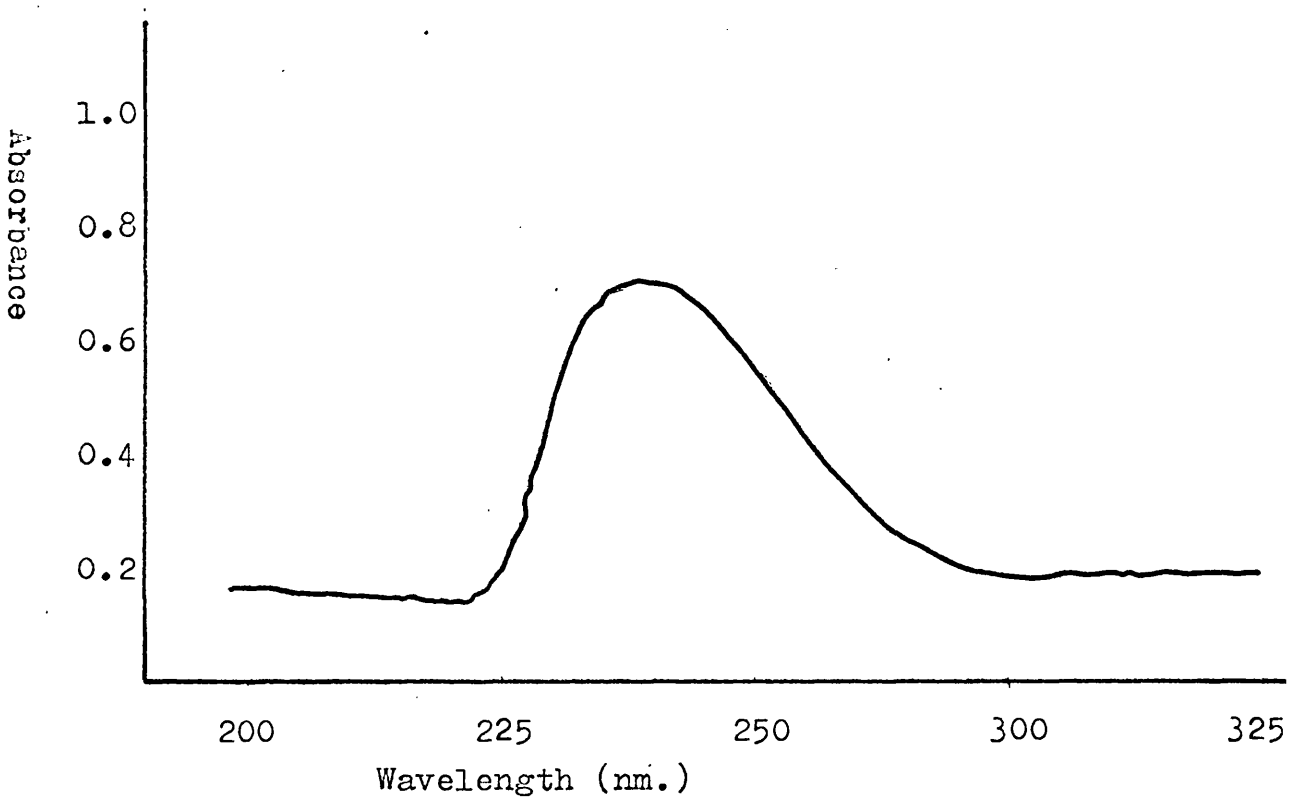
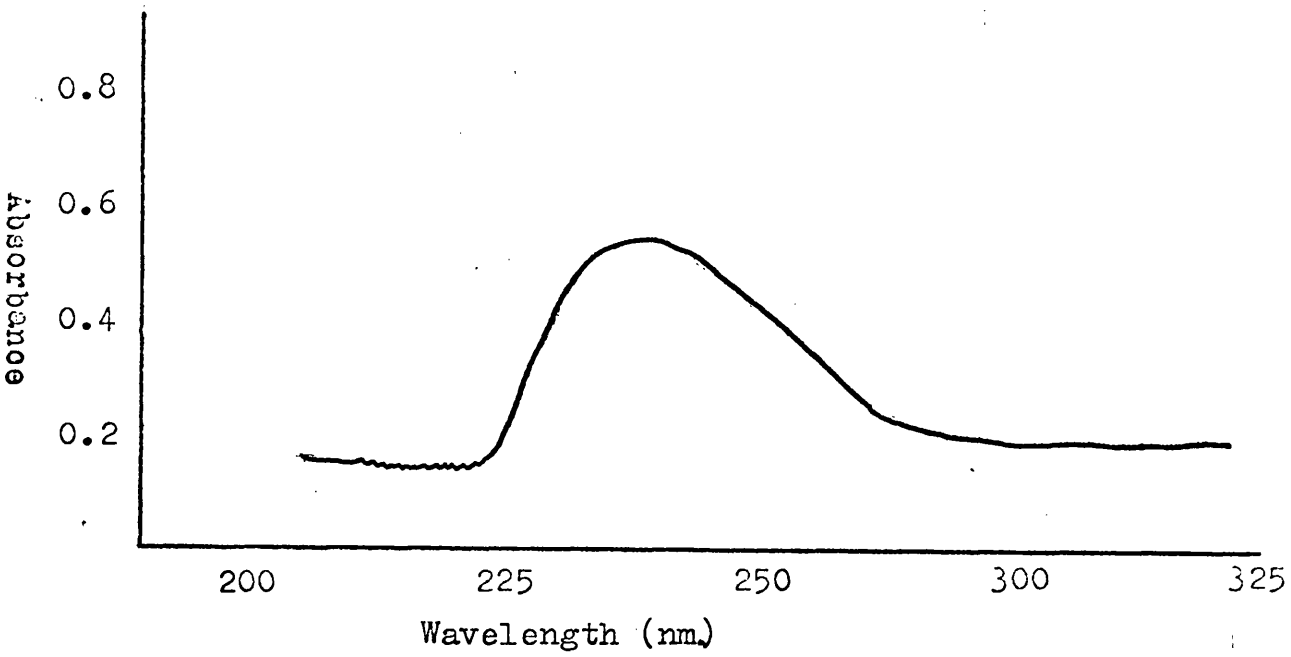


Fig 17. Absorption of the Reaction Products of P.T.E. from Penicillium italicum and Pectin at pH 5.2. (traced from original scan).



on pectin of unknown molecular weight, it is necessary to take an approximate value for this. Molecular weights for pectin have been reported to vary from 20,000 to 200,000. Thus as the pectin used in the present work was an extracted material, and was likely to have been considerably deploymerised during extraction, it was decided to take the lower value as the approximate molecular weight for the calculation of Km. The Michaelis constants obtained by this method are not therefore absolute values, but they can be used to compare the P.T.E.s from each organism. The activity of each P.T.E. was thus measured at a variety of substrate concentrations and the results obtained are shown in table XII and Figs 18 and 19. From these the apparent Km value for both P.T.E.s was 0.003 milli molar.

Table XII. The Effect of Substrate Concentration on P.T.E. Activity

Substrate Conc.(%)	Substrate Conc.(milli molar)	Reciprocal of Substrate Conc.	Reciprocal of P.T.E. Activity.			
			P.digitatum		P.italicum	
			a.	b.	a.	b.
0.5	0.025	40	0.146	0.173	0.194	0.199
0.4	0.020	50	0.146	0.176	0.216	0.211
0.3	0.015	67	0.186	0.207	0.272	0.260
0.2	0.010	100	0.270	0.289	0.379	0.356
0.1	0.005	200	0.457	0.490	0.662	0.667
0.05	0.0025	400	0.719	0.775	1.042	1.020
0.01	0.0005	2000	4.167	3.704	4.546	5.000

Fig 18. 1/v : 1/S Plot for the P.T.E. from Penicillium digitatum.

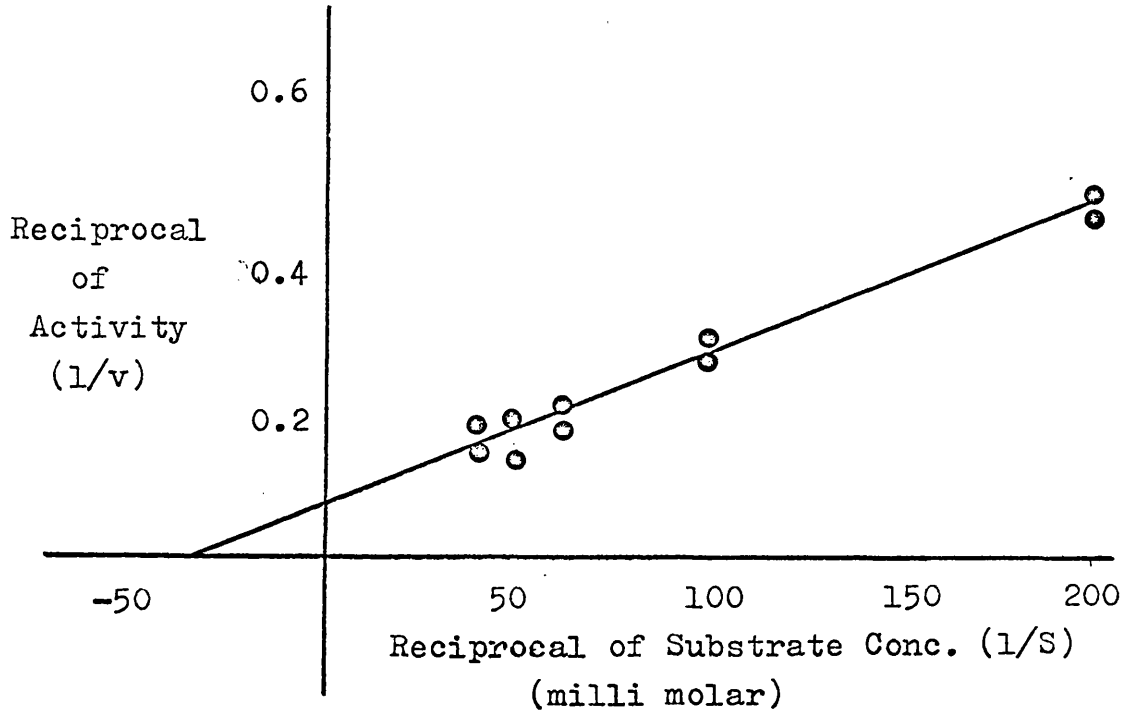
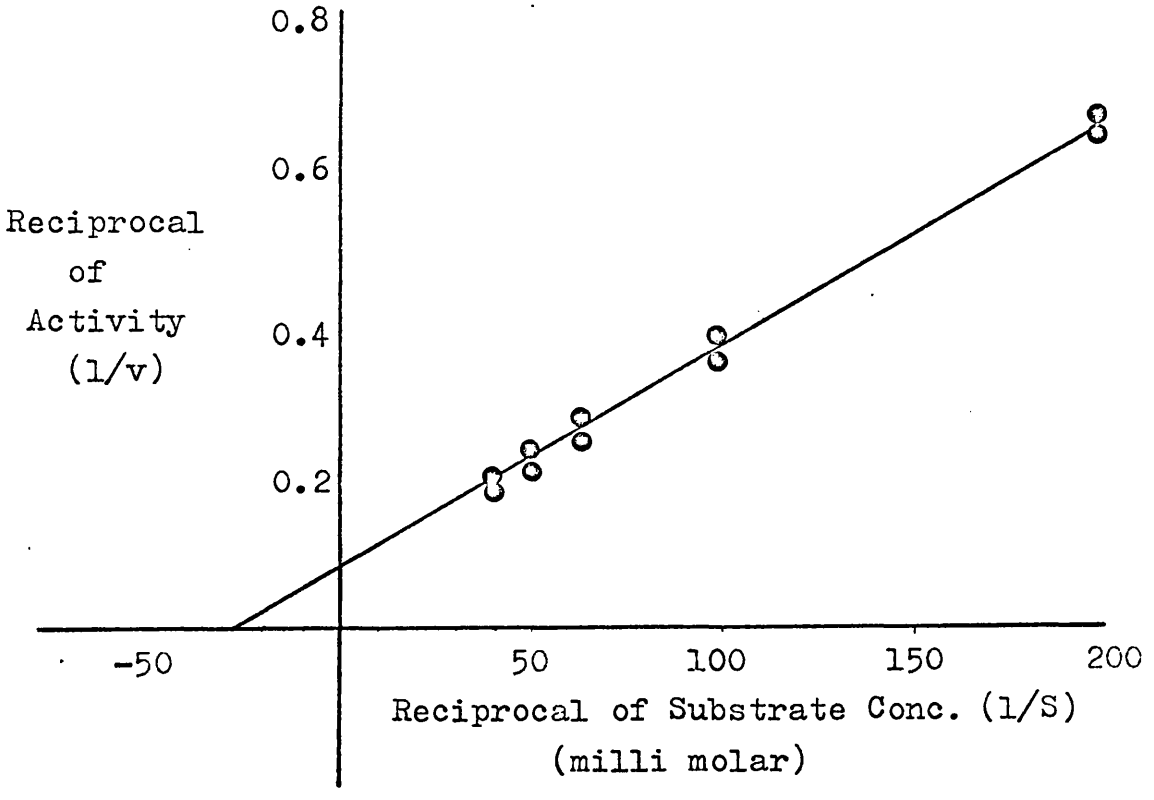


Fig 19. 1/v : 1/S Plot for the P.T.E. from Penicillium italicum.



4. Effect of pH on P.T.E. Activity. Activity of the two P.T.E.s was measured over a range of pH. The optimum pH for both enzymes was about 5.5. This is higher by 0.3 pH units than the reported value for P.T.E. of 5.2. (Albersheim and Killias 1962). The effects of pH on enzyme activity are shown in Figs 20 and 21.

5. Thermal Inactivation of P.T.E. 0.5ml samples of undiluted, purified P.T.E. were held at temperatures ranging from 50 to 75°C, for periods of 5 minutes. Preliminary experiments had indicated that inactivation occured in this temperature range. At the end of the immersion period, the 0.5ml samples were removed from the water bath and diluted to 5ml with distilled water at room temperature. This ensured that the samples were maintained at the inactivation temperature for exactly 5 minutes. Controls were treated in the same way, except that they were kept at room temperature continuously. The results are shown in table XIII. Percentage reduction in activity was calculated by the formula:- $R = \frac{C - A}{C} \times 100$ where:-

R = % age reduction in activity
C = activity of control
A = activity of sample after immersion

There is as can be seen from the results, a distinct difference between the thermal stabilities of the two P.T.E.s. Penicillium italicum P.T.E. is inactivated at a lower temperature than the P.T.E. produced by Penicillium digitatum. The P.italicum P.T.E. was also inactivated more than the P.digitatum P.T.E. during the purification process. It is therefore possible that there is a structural difference between the enzymes, although the variations in thermal stability might be explained by the presence of traces of impurities

Fig 20. Relationship between pH and Activity of P.T.E. from Penicillium digitatum.

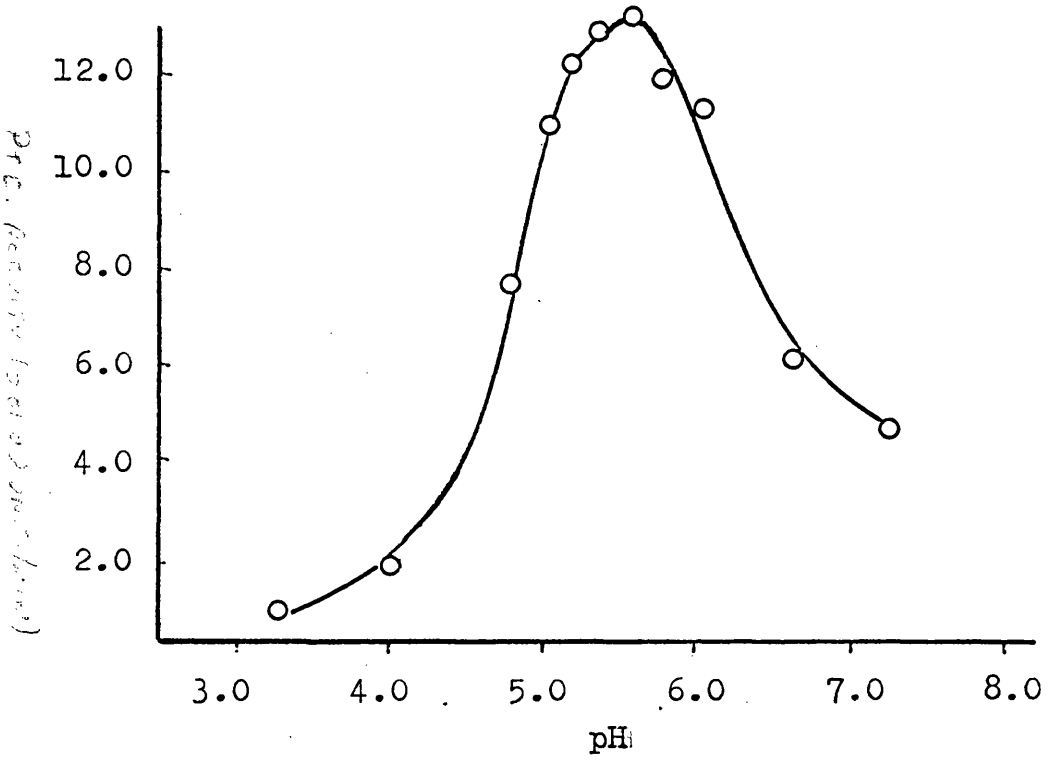
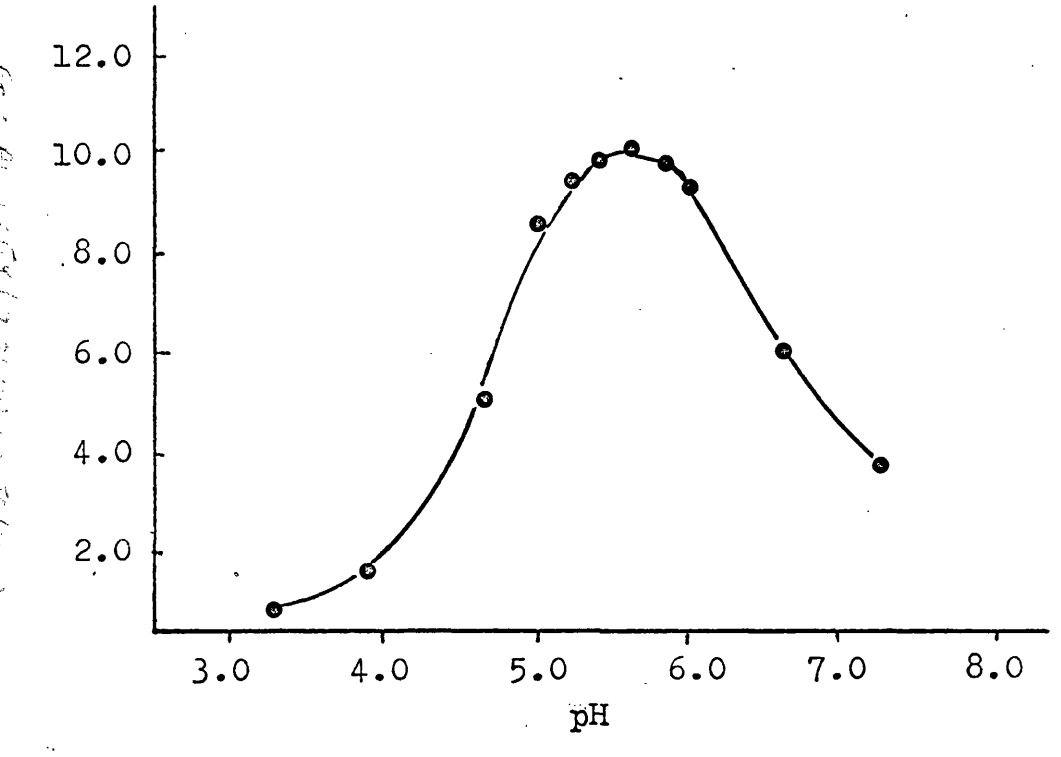


Fig. 21. Relationship between pH and Activity of P.T.E. from Penicillium italicum.



other than proteins, in the electrophoretically homogeneous preparations.

Table XIII. Thermal Inactivation of P.T.E. from Penicillium digitatum and Penicillium italicum

Temp. °C Samples held at this for 5 mins.	Penicillium digitatum P.T.E.		Penicillium italicum P.T.E.	
	Activity	%Reduction in Activity	Activity	%Reduction in Activity
50	3.17	2.2	2.77	11.8
55	3.14	3.1	1.81	42.4
60	1.98	38.9	0.19	93.9
65	0.11	96.6	0.02	99.4
70	0.0	100.0	0.0	100.0
75	0.0	100,0	0.0	100.0
Control	3.24		3.14	

6. Effect of pH on the Pectin Viscosity Reducing Activity of the P.T.E. Preparations. Previous experiments had indicated that P.T.E. was responsible for the reduction in viscosity of pectin solutions, caused by filtrates of Penicillium digitatum and Penicillium italicum. It was also found that the electrophoretically homogeneous P.T.E. preparations from both organisms were able to cause a decrease in viscosity of pectin

solutions. To obtain further confirmation that P.T.E. was responsible for viscosity reducing activity, the action of the purified P.T.E. preparations on the viscosity of pectin, was examined over a range of pH. The activity pH curves, obtained in this experiment (Figs 22 and 23.) are of the same form as the activity pH curves found when P.T.E. activity was measured spectrophotometrically. The optimum pH for both types of activity differed by less than half a pH unit. The apparent ability of the P.T.E.s from both organisms to reduce the viscosity of pectin, points very strongly to their being endo enzymes. An exo enzyme would be unlikely to have enough effect on the molecular size of pectin, to cause a reduction in viscosity. This in turn substantiates evidence so far put forward, that P.T.E. can be responsible for maceration of orange tissue, as it is recognised (Bateman and Millar 1966) that endo enzymes are more likely to be responsible for the maceration of plant tissues than exo enzymes.

7. Effect of pH on the Maceration of Orange Tissue by the Purified P.T.E. Preparations. Although it is realised that the macerating activity assay used in the present work is only partially quantitative, an attempt was made to determine the effect of pH on maceration, within the limits of the maceration assay. Four ml of 0.1 M McIlvaines citrate phosphate buffer were added to 1ml of undiluted, purified P.T.E. preparation, and two orange discs were placed in this. Softening of the discs was estimated after 1 and 24 hours at the following pH values:- 3.0, 4.0, 5.0, 5.5, 6.0, 7.0, and 8.0. Orange discs were also placed in buffer at the various pH values in order to determine how much softening was caused by the buffer alone. The results are shown in table XIV.

Fig 22. Relationship of pH with Viscosity Reducing Activity on Pectin of P.T.E. from Penicillium digitatum.

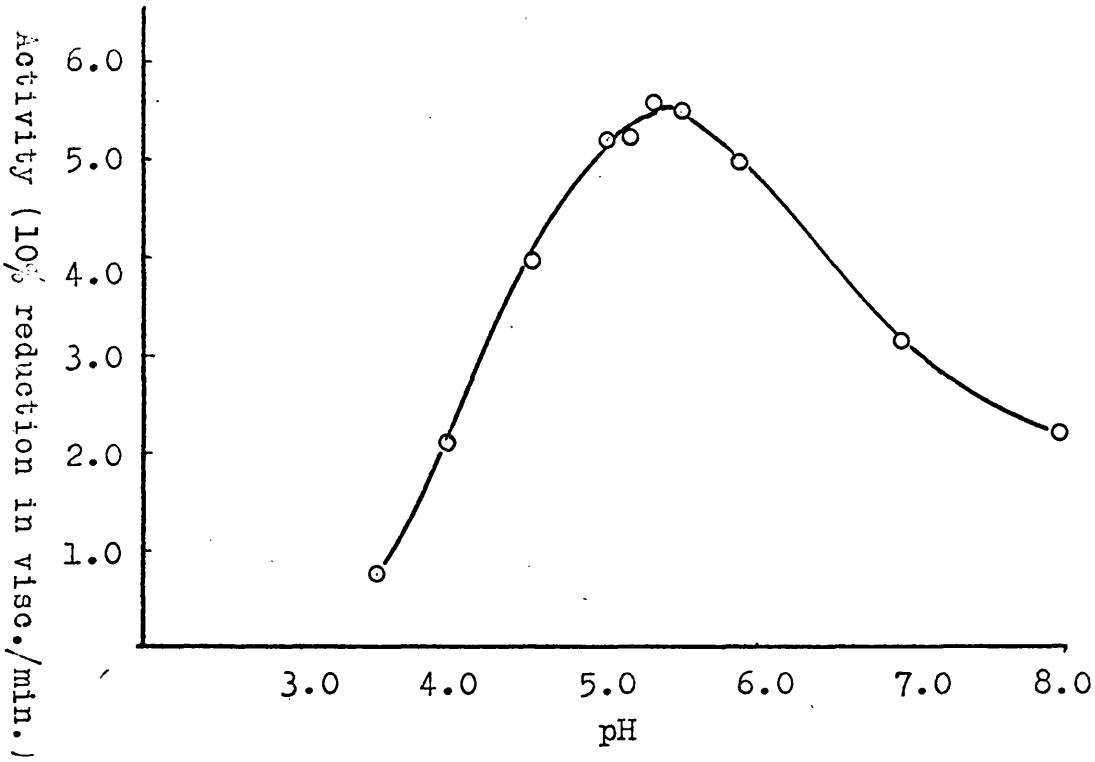


Fig 23. Relationship of pH with Viscosity Reducing Activity on Pectin of P.T.E. from Penicillium italicum.

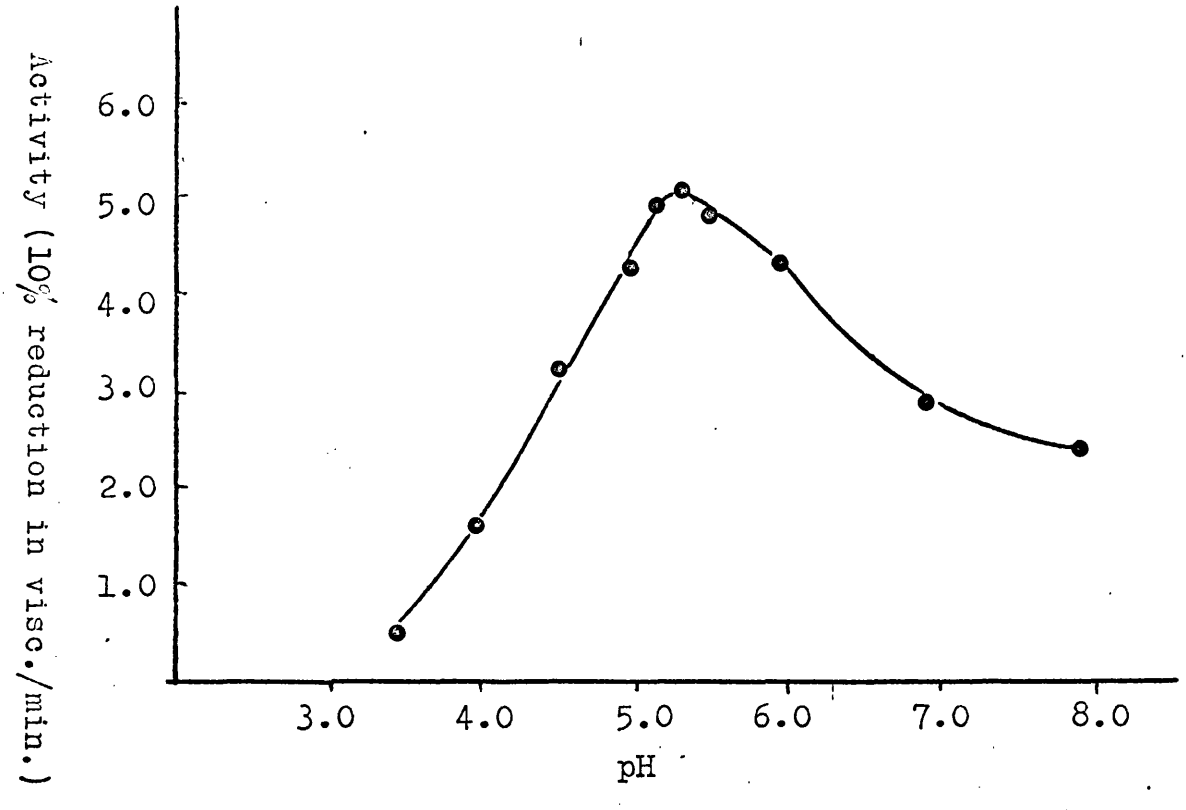


Table XIV. The Effect of pH on Macerating Activity of the Purified P.T.E. Preparations.

pH	Penicillium digitatum P.T.E.		Penicillium italicum P.T.E.		Controls (buffer without enzyme)	
	1 hr.	24 hr.	1 hr.	24 hr.	1 hr.	24 hr.
3.0	±	++	-	++	±	++
4.0	+	+++	±	+++	±	+
5.0	+++	+++++	++	++++	-	±
5.5	+++	+++++	++	++++	-	±
6.0	+++	+++++	++	++++	-	-
7.0	±	++	+	++	-	-
8.0	±	+	±	+	-	-

In view of the softening which occured in the controls, at pH 3.0 and 4.0, it is questionable how much softening is due to enzyme activity and how much to pH, in the course of the rotting of fruits by Penicillium digitatum and Penicillium italicum. The pH of orange rind, macerated in water (wt./vol. 1:1), is between 4.0 and 4.5, thus both enzymic and pH maceration could theoretically occur.

8. Action of P.T.E. on Pectic Acid. Pectic acid transeliminase was not detected in culture filtrates of the 3 organisms examined, in the course of the enzyme production experiments. However, during the fractionation of the culture filtrates, a small amount of pectic acid transeliminase (P.G.T.E.) activity was detected in fractions containing P.T.E. It therefore seemed likely that P.T.E. had a very limited action on pectic acid. In order to substantiate this theory, the action of the purified P.T.E. samples on pectic acid was investigated over a range of pH values. The level of activity found was so small that it was not practicable to represent this graphically; the results are therefore shown in table XV. P.G.T.E. activity was only found at pH values where P.T.E. was most active (between pH 4.5 and 6.0). It is therefore considered most likely that none of the organisms investigated produced a P.G.T.E., and that any such action recorded, was entirely due to the action of P.T.E. (Aspergillus fonsecaeus produced so little P.T.E. in any case, that transeliminative activity on pectic acid was never recorded). From the results it appeared that P.T.E. attacked methylated pectin at about 500 times the rate which it attacked pectic acid.

9. Chromatography of Reaction Products of P.T.E. Acting on Pectin. Samples of P.T.E. from Penicillium digitatum and Penicillium italicum were mixed with 0.5% pectin in 0.1 M citrate phosphate buffer at pH 5.2. and after 1, 12 and 24 hours part of the reaction mixture was applied to a thin layer chromatogram. Galacturonic acid and pectin were used as markers and the enzyme and buffer alone was applied as a control. No galacturonic acid was detected in the reaction mixtures, though a trace of material between the markers of pectin and

galacturonic acid was observed. This was probably one of the smaller oligomers of unsaturated galacturonic acid. The results give further indication that both P.T.E.s are endo enzymes.

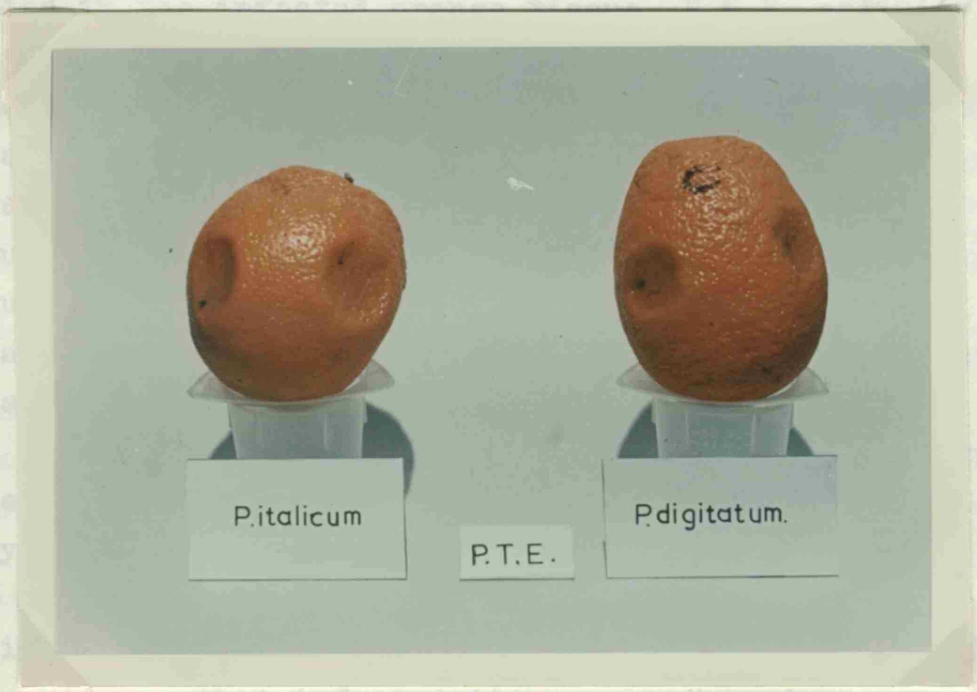
Table XV. Action of the Purified P.T.E. Preparations on Pectic Acid, over a Range of pH Values.

pH	Pectic Acid Transeliminase Activity (0.01 O.D. units/min)	
	Penicillium digitatum P.T.E.	Penicillium italicum P.T.E.
3.5	0	0
4.0	0	0
4.5	0.02	0.03
5.0	0.05	0.04
5.2	0.05	0.03
5.4	0.04	0.04
5.6	0.04	0.03
5.8	0.04	0.04
6.0	0.07	0.02
7.0	0	0
8.0	0	0

10. Softening of an Intact Orange with Purified P.T.E. Oranges were injected with 1ml of purified P.T.E. (about 400 units of activity), from both Penicillium digitatum and Penicillium italicum. One ml portions of boiled enzyme were injected as controls. After 1 hour, slight softening was noticed around the sites of injection of the active enzymes, but not around the sites

of injection of the boiled enzymes. After 12 hours the the softening became more apparent, with the softened area turning brown and becoming sunken. The fruits with these softened areas dried out more quickly than the control fruits. The area of softening remained discrete, and the diameter varied from about 2 to 3 cms. This suggested that the enzyme was absorbed onto the orange tissue fairly quickly and was therefore prevented from diffusing over a wide area. Alternatively the discrete areas of softening may be due to the enzyme being inactivated by substances in the rind tissue. The effects of the purified enzymes injected into oranges are shown in plate 2.

Plate 2. Oranges Injected with Purified P.T.E.



g. Production of Enzymes in Vivo by Penicillium digitatum and Penicillium italicum.

Oranges infected with Penicillium digitatum and Penicillium italicum, and non infected oranges were incubated for 5 days at 25°C. At the end of this time the peel from all the oranges was removed, and 50g of peel from each treatment was macerated with 100ml of 0.5 M $Na_2H.PO_4$ at about pH 8.0, and stirred for an hour. The macerates were then centrifuged and the supernatants were made up to 0.8 saturation with ammonium sulphate. The precipitates so formed, were centrifuged at 12,000 rev/min. (23,000 g) for 20 minutes, and were then made up to 50ml with distilled water. Enzyme assays were then carried out on these solutions as shown in table XVI.

P.T.E. activity, pectin viscosity reducing activity, P.M.E. and macerating activity were demonstrated in the infected orange tissue. P.M.E. activity was also demonstrated in the healthy orange tissue. Although arabanase activity was not demonstrated, evidence that such activity had been present in infected tissue was obtained, as extracts from this tissue had a higher pentose content than the healthy tissue extract. It is considered likely that the arabanase was inactivated by the high pH of the extracting agent ($Na_2H\ PO_4$)

Considerable difficulty was experienced at first in demonstrating any activity in vivo. Initially activity was shown by dispersing infected peel in a 0.5% pectin solution at pH 5.2 and observing the drop in viscosity of this mixture after centrifugation. With such evidence that infected tissues contained enzyme activity, various extraction methods were attempted. It is considered that with more investigation into

extraction proceedures, greater in vivo activity could be demonstrated. It is also probable that enzymes not demonstrated in this work, could be shown to be present by using different and improved methods of extraction.

Table XVI. Enzymes Present in Extracts of Healthy and Infected Oranges.

Enzyme Assay. (activity units as described previously)	Extract of Healthy Fruit	Extract of Fruit infected with P.digitatum	Extract of Fruit infected with P.italicum
P.T.E.	0	0.27	0.31
Viscosity Reducing Activity on Pectin	0	0.18	0.16
Arabanase	0	0	0
Liberation of Reducing Groups from Pectic Acid	0	0	0
P.M.E.	+	+	+
Maceration			
1 hr.	-	±	±
24hrs.	-	++	++

DISCUSSION

Penicillium digitatum and Penicillium italicum have been shown to produce P.T.E., macerating factor, and the ability to reduce the viscosity of pectin, under a variety of conditions, including the in vivo state. Arabanase was also produced in culture media, but it was not shown in extracts of infected orange peel. Evidence that arabanase had been active in infected fruits was obtained. P.M.E. was produced by both organisms under all conditions, but it was also present in healthy orange tissue. P.G., as measured by the liberation of reducing groups from pectic acid, was detected under certain culture conditions, but was not shown to be present in extracts of infected orange rind tissue. The fact that Penicillium notatum, an organism not pathogenic on citrus fruits, produced P.M.E. and P.G. as detected by plate assay, was taken as evidence for the view that these enzymes were unlikely to be concerned in the initial invasion of citrus fruits by the pathogenic moulds. Macerating activity was produced under the same conditions as P.T.E., throughout the experiments on growth conditions, and when purified, P.T.E. from both P.digitatum and P.italicum, was found to cause maceration of orange rind tissue. The two P.T.E.s were compared electrophoretically and were found to have the same mobilities, although at present electrophoresis has only been carried out at one pH value. Apart from causing maceration, both purified P.T.E.s were able to reduce the viscosity of pectin. Their pH : activity curves when measured by, a. the increase in optical density at 235 nm of a pectin solution, b. the reduction in viscosity of a pectin solution and c. the maceration of orange tissue, were all similar; the pH

optima recorded with a., b., and c., were all of the same order.

The P.T.E.s from Penicillium digitatum and Penicillium italicum, were shown by thin layer chromatography to be endo enzymes. They were found to be similar in all respects, apart from their thermal stability. P.italicum P.T.E. was inactivated at a lower temperature than the P.T.E. of P.digitatum. At present no explanation can be put forward for this discrepancy, although conclusive evidence that it was a discrepancy rather than a real difference between the enzymes, could perhaps be obtained, by carrying out electrophoresis of the purified samples at several pH values, or by further purifying the samples before carrying out the thermal inactivation tests on them. Finally, when samples of the purified enzymes were injected into whole oranges, they caused areas of softening in the rind, whereas boiled enzyme did not. The results obtained are significant on three accounts. Firstly they shed further light on the attack of citrus fruits by P.digitatum and P.italicum. Secondly they support certain evidence on the structure of plant cell walls, and thirdly they add to the general knowledge of the role of pectic enzymes in host parasite relationships.

P.G. and P.M.E. were shown to be constitutively produced by both Penicillium digitatum and Penicillium italicum (Miyakawa 1962, Kavanagh 1965.), whereas only P.M.E. was found to be produced under all conditions in the present work. However, as these workers were using different media, it was difficult to make a comparison. It did appear however from the present results that the production of P.G. was dependent on the nature of the medium (bran strongly favoured its production)

and aeration conditions, rather than its pectin content. Miyakawa (1963), also suggested that D-galacturonic acid, identified by him in infected citrus peel, might have a role in the maceration of orange tissue. Although no direct evidence for this has been found in the present work, it has been observed that maceration of orange tissue can occur in enzyme free buffer below about pH 4.5.

Much of the work carried out on enzyme production by Penicillium digitatum, has been done by Cole (1967). Although his results are not in complete agreement with the present work, no serious problem is encountered in reconciling the two groups of observations. Enzyme production by P.digitatum was examined under a wide variety of conditions by Cole, whereas in the present work, only three media, all different from those used by Cole, were employed. Comparison from this point of view is therefore difficult, but evidence from both sets of results has suggested that arabanase is not principally concerned in maceration. The present work differs mainly from that of Cole, in that he casts doubt on the suggestion that, the P.T.E. component of the pectic enzyme complex produced by P.digitatum is responsible for maceration. The evidence presented by Cole in support of this view, is that maceration has been shown by him, to be produced under conditions of growth, where he was unable to detect P.T.E. In no case however, did he report that macerating activity was absent, when P.T.E. was present. The results of the fractionation of P.digitatum culture filtrate (tables VI and VII), clearly show that macerating activity was associated with P.T.E., and this view is borne out by the fact that the electrophoretically homogeneous P.T.E. preparation from P.digitatum, was able to macerate

orange tissue. Further to this, no other enzyme activity was detected in such pure preparations. However it is not denied that more than one macerating system may operate in P.digitatum culture filtrates, produced under varying medium and culture conditions. Strong evidence of an alternative macerating system, has been found using fractions of culture filtrates of P.italicum low in, or free from P.T.E.; and in the case of Aspergillus fonsecaeus it seems probable that the amount of maceration caused by P.T.E. is of limited significance. However the results given above, strongly indicate that in culture conditions where P.T.E. is produced, the presence of this enzyme can account for all or part of the macerating activity of the filtrate.

A further contrast between the results found by Cole (1967) and those presented in this work, occured in the detection of enzymes in infected orange tissue. P.T.E., the ability to reduce the viscosity of pectin, macerating activity and P.M.E. have been detected in vivo in the present work, whereas P.G. and arabanase have not. Cole was unable to detect P.T.E. in vivo, although he detected a small amount of viscosity reducing activity. The detection of enzyme activity in infected tissue is difficult for two reasons. Firstly the enzymes may be inactivated by substances in the tissue. For example,Cole himself found that orange juice contained a thermolabile inhibitor to macerating activity and viscosity reducing enzymes, and Cole J.S. (1956), found that oxidised apple juice was inhibitory to the pectic enzymes of Botrytis cinerea. Secondly enzymes can become absorbed strongly to the host tissue, when vigorous methods have to be used to remove them. This in itself may result in inactivation of the enzymes. A further difficulty is experienced in

the demonstration of P.T.E. in infected orange peel, due to the high U.V. absorption of the extract. The sensitivity of the spectrophotometer is then inadequate to detect small increases in absorption. This difficulty was overcome by reading the optical density of reaction mixtures against a blank with a high U.V. absorbance and by using an ammonium sulphate precipitate of the extract, rather than the extract itself, to detect activity. When these difficulties are taken into consideration it is suggested that, the inability to demonstrate the presence of an enzyme in vivo, is not conclusive evidence that this enzyme is not produced under such conditions. This suggestion is emphasized by the work of Garber and Beraha (1966), who demonstrated both an exo and an endo-P.G. in infected orange tissue, in contrast to the results presented here. On the evidence therefore, that both Penicillium digitatum and Penicillium italicum produce P.T.E. in vitro and in vivo; and that when purified, this enzyme will macerate orange tissue and cause softening of the rind of intact oranges, if injected into them, it is suggested that P.T.E. has a very significant role to play in the host parasite relationships of these fungi and citrus fruits.

That other pectic enzymes have an important role in this host parasite relationship, either specifically in maceration or in pathogenicity in general is clear from the work of others (Cole, 1967; Garber, Beraha and Shaeffer, 1965; Kavanagh, 1965.). It is considered likely that endo-P.G. may have a role in maceration together with P.M.E.; but that other enzymes, eg. arabanases and exo-P.G. may act on the breakdown products of the chain splitting enzymes, to produce monomeric forms that can be absorbed by

pathogens. These substances will then presumably take part in the metabolism of the fungus. From the results presented here, there appears no need to invoke a macerating system in either Penicillium digitatum or Penicillium italicum, which does not involve either endo-hydrolases or endo- transeliminases breaking down pectin or pectic acid, as postulated by Cole (1967). Evidence of such an unknown system has however, been found in the case of Aspergillus fonsecaeus, where maceration has been detected in filtrates containing no pectic enzymes.

In more general terms of host parasite relationships, the results lend further weight to the argument of McClendon (1964), that pectic materials are of fundamental importance in the structure of the middle lamella of plants. The P.T.E. of Penicillium digitatum. was found to be capable of causing maceration of not only orange tissue, but also rose leaves. The rose leaves presented a more suitable substrate for microscopic examination, than the rather heterogeneous tissue of orange peel. When a rose leaf was placed into a solution of enzyme, it gradually disintegrated. If then the suspension of macerated leaf tissue was examined microscopically, it was found that the enzyme had caused a complete separation of the cells, although the cells themselves, remained intact for several days. It is thus significant that P.T.E., an enzyme having a specific action on extracted pectin, was able to cause a disintegration of plant material, by breaking down plant pectin. This gives support to the view of Joclyn (1962), who considered that protopectin (plant pectin) was not a substance basically different in structure from extracted pectin The results of the present work can be reconciled either with the view

that it is the size of the pectin molecule, which renders it insoluble in plant tissue, or that it is the way in which the molecule is bound with other substances in the tissue that is the cause of its insolubility.

The results of the present work satisfy the conditions laid down by Wood (1960), to prove that pectic enzymes play a part in plant disease. These are that such enzymes are, or have been present and active in infected plant tissue, as is the case with P.T.E. in the present investigation. The results are also in agreement with the main conclusions of Bateman and Millar (1966), which were that maceration was most likely to be correlated with the endo forms of both hydrolases and transeliminases, although it was admitted that other enzymes might play a part in pathogenicity. As has been mentioned, a certain amount of evidence was obtained in the present work, for the involvement of endo-P.G. in maceration in the case of Penicillium italicum. What has not been obtained in work up to the present time, in Bateman and Millar's view, is proof that the ability of an organism to produce pectic enzymes, is sufficient to account for its pathogenicity. Although it is not claimed that such proof has now been obtained, it is felt that strong evidence for this view has been put forward. This evidence is based on the observation that Penicillium digitatum and P.italicum will both invade oranges wounded with pin pricks, whereas Penicillium notatum will not. P.notatum will however, grow on the surface of a cut orange, but will not soften the fruit or grow throughout the tissue. It thus appears that the ability of the pathogens to spread through the orange tissue, is connected with their ability to cause softening of the fruit. In addition

to this, strong evidence that the softening is caused by pectic enzymes (in particular P.T.E.), has been presented in these results. Further proof that the pathogenicity of P.digitatum and P.italicum on citrus fruits depends on their ability to produce P.T.E., might be obtained by using a specific inhibitor to P.T.E., if such a substance could be obtained. If an orange treated with this inhibitor was found to be immune to attack by the two pathogens, the importance of P.T.E. would have been shown.

The P.T.E.s produced by Penicillium digitatum and Penicillium italicum appear to be identical in all respects, apart from their thermal inactivation temperatures. They resemble the transeliminase isolated by Albersheim, Neukom and Deuel (1960), although the absorption peak of the reaction mixture using this enzyme was reported to be 235 nm., whereas a slightly higher value (239 nm.) has been found in the present work. The P.T.E. isolated by Edstrom and Phaff (1964a.), from Aspergillus fonsecaeus, appeared to resemble the P.T.E.s of P.digitatum and P.italicum. However when the A.fonsecaeus P.T.E. was examined in the present work, it was found to behave differently on ion exchange cellulose, from the P.T.E.s produced by the two Penicillia. In general fungi have been shown to produce transeliminases attacking pectin (Albersheim and Killias, 1962; Edstrom and Phaff, 1964), and bacteria have been shown to produce transeliminases attacking pectic acid (Nagel and Vaughn, 1961; Fuchs, 1965.). An exception to this situation, is the transeliminase isolated by Hasegawa and Nagel (1967), from an aerobic Bacillus, which was able to attack both pectin and pectic acid. The transeliminases from P.digitatum and P.italicum, however, appeared to follow the general rule, in that they attacked only pectin

significantly. No pectic acid transeliminase was detected in either of these fungi, nor in A.fonsecaeus.

Apart from providing evidence for the view that P.T.E. and macerating factor in Penicillium digitatum and Penicillium italicum, are synonymous, and that these enzymes play a significant role in pathogenesis, the present work has also suggested several possible lines for further research. Such research could follow either an applied course or an academic one. Applied research could be carried out with the aim of obtaining a specific inhibitory agent to P.T.E., which, if non-toxic, might be used to control the green and blue rots of citrus fruits. At the moment, di-phenyl impregnated wrapping paper is used in an attempt to control these diseases. This is unsatisfactory both from the toxicity point of view, and in its efficiency in controlling the diseases. Thus the develop ment of an improved antifungal agent, for use in controlling the Penicillia rots of citrus fruits, would be a worthwhile research aim.

The method used in this work for the purification of P.T.E. is both quick and simple. It can be envisaged therefore, that pure P.T.E. might have a use in the fruit juice industry. At the present time a mixture of P.M.E. and P.G.,in crude extracts, is used to reduce the viscosity of fruit pectins in the manufacture of fruit juices. The disadvantages of this system are twofold; firstly the rate of the viscosity reduction is limited by the rate of P.M.E. activity, and secondly, the crude extract has to be used in relatively large quantities, risking the addition of undesirable impurities to the juice. Both disadvantages might be overcome by using a purified P.T.E. preparation of the kind obtained in this work.

Work of a more academic nature might involve the study of the products of transeliminase action. The double bond between carbon atoms 4 and 5 of the galacturonic acid residues should theoretically render these structures unstable. It would be of interest therefore, to examine their fate in the metabolism of the fungi, if indeed they are absorbed without further degradation. It is possible to envisage the conversion of these unsaturated structures to galacturonic acid by a separate enzyme system(s), although no evidence has been found for such a system at present.

As comparatively little is known about P.T.E. at the present time, further studies on its substrate specificity would be of interest. This would involve the preparation of a series of oligomers of saturated and unsaturated galacturonic acid, which could undoubtedly be carried out, using a range of Sephadex gels. A great deal could then be learnt about the mode of action of P.T.E., using these defined substrates and a purified enzyme preparation

Finally the nature of other enzymes systems responsible for maceration, might be investigated further. Cole (1967), found evidence of such systems in Penicillium digitatum, and the results shown above have presented evidence of macerating systems other than P.T.E. in Penicillium italicum and Aspergillus fonsecaeus. Geotrichum citri-auranti has been reported to invade citrus fruits (Fawcett, 1926). However, apart from ascertaining that the organism will do this, no work has been carried out on its pathogenicity of citrus fruits. Thus an investigation of the pectic enzymes produced by this organism, and a comparison of these with the enzymes produced by

P.digitatum, P.italicum and A. fonsecaeus, might prove very fruitful .

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